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(54) Title: ANTIGEN–mPEG CONJUGATES SUPPRESS HUMORAL AND CELL MEDIATED IMMUNE RESPONSES

(57) Abstract

A method of inducing tolerance to an antigen (Ag) in both humoral and cell–mediated immune responses by administering an effective amount of an immunosuppressive Ag(mPEG) conjugate. Methods of treating allergies, autoimmune diseases and preventing an immune rejection of organ transplants and DNA transfected cells or cells transfected with a gene therapy vector encoding a foreign protein for gene therapy are also disclosed.

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ANTIGEN-mPEG CONJUGATES SUPPRESS HUMORAL AND CELL MEDIATED IMMUNE RESPONSES

Technical Field

The present invention relates to a method of inducing tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses by administering an effective amount of an Ag conjugated with monomethoxypolyethylene glycol (mPEG) conjugate. Methods of treating allergies, autoimmune diseases and preventing an immune rejection of organ transplants and rejection of genetically engineered cells in gene therapy, are also disclosed.

Background Art

Numerous studies have shown that exogenous protein Ag can activate $CD8^+$ T cells that suppress immune responses in an Ag-specific fashion (1-5). These observations have been puzzling because exogenous Ag normally are not processed via the endogenous pathway for major histocompatibility complex (MHC) class I presentation in most somatic cells. For example, nonphagocytic EL4 cells do not present soluble ovalbumin (OVA) to MHC class I-restricted $CD8^+$ T cells after pulsing with native OVA (6). However, exogenous OVA could be presented via MHC class I pathway by these cells if delivered by other means, such as osmotic lysis (6), electroporation (7), liposomes (8), mycobacterial infection (9), nonionic triblock copolymers (10) or receptor-mediated uptake (11).

The observations that protein Ag(s) alone generally do not prime $CD8^+$ cytolytic T cells (CTL) *in vivo* have been taken as evidence that exogenous Ag do not stimulate $CD8^+$ T cells. Whether $CD8^+$ CTL precursors are activated by exogenous Ag administered with traditional adjuvants is more controversial (12). Nevertheless, the inventors have previously shown that OVA emulsified in adjuvants, such as CFA or nonionic triblock copolymers, primed OVA-specific,

MHC class I-restricted $CD8^+$ CTL precursors *in vivo* (4,13). Priming was not due to direct sensitization of MHC class I-bearing cells by contaminating peptides; instead, phagocytic cells were required for priming $CD8^+$ CTL (4). These findings indicated that exogenous Ag were taken up and processed via the MHC class I pathway by phagocytic macrophages, as suggested by Rock et al. (14).

Previous data have shown that adoptive transfer of Ag-specific $CD8^+$ CTL suppressed subsequent Ab responses in recipients (4,15). Thus, activated CTL inhibit Ag-specific immune responses *in vivo*, as had been shown *in vitro* (2,16). Clearly, such T cells could account for some of the activities attributed to $CD8^+$ Ts cells. More recently, the inventors found that oral administration of soluble protein Ag stimulated $CD8^+$ Ts cells that inhibited humoral responses and priming of both $CD4^+$ and $CD8^+$ T cells (5). These $CD8^+$ Ts cells were not CTL and phenotypically distinguished from $CD8^+$ CTL by reacting with a mAb specific for Ts cells (17).

Published PCT application WO 95/12413 mentions T cells, and even cytotoxic T cells, as potential targets for inhibition by mPEG compounds. In the PCT, various proteins, such as OVA (ovalbumin) or IgG, were chemically coupled with mPEG. At the appropriate degree of conjugation of mPEG, some of the epitopes of the proteins are still accessible for interaction with the appropriate specific, antibody and this provides the means to target these molecules to B cells or to granulocytes that have bound IgE to their surface via Fc receptors.

Unlike antibodies, the antigen-specific receptors expressed by T cells do not recognize native, intact proteins. Rather, they bind to protein fragments that are bound by proteins encoded by the Major Histocompatability Complex expressed on the surface of specialized antigen presenting cells. The PCT publication WO 95/12413 does not propose that mPEG antigens would be used to inhibit T cells.

This treatment would be non-antigen-specific. Such nonspecific inhibition would have little advantage over the immunosuppressive drugs that are currently in use.

The PCT publication has demonstrated that mPEG modification of proteins renders them non-immunogenic in that they fail to stimulate antibody responses. More importantly, the mPEG modified proteins are tolerogenic rendering exposed individuals unable to respond to a subsequent challenge with the unmodified, protein. Thus, tolerance induced by mPEG modified proteins is due to induction of suppressor T cells that in turn down regulate in an Ag-specific manner the immune response of the recipient if administered at any time prior to challenge with the unmodified antigen. Sehon has obtained several patents on a procedure for preventing IgE mediated allergic responses, and favoring the tilting of the immune response to the production of IgG antibodies in allergic individuals. However, the effects of mPEG modified proteins on cell-mediated immune responses have only recently been studied.

U.S. Patent No. 4,296,097 discloses a process for the suppression of the formation of anti-BPO (benzylpenicilloyl) antibodies by administering a conjugate of penicillin and an amino-derivative of polyvinylalcohol polymers.

U.S. Patent No. 4,261,973 discloses a method of suppressing the induction of reaginic antibodies to an allergan by administering a covalent conjugate of the allergen and non-immunogenic water soluble polymers.

U.S. Patent No. 5,447,722 describes a method of suppression of an IgG immune response to an antigenic protein by administering a tolerogenic conjugate of monomethoxypolyethylene glycol and the antigenic protein one day prior to adminstration of the protein alone.

U.S. Patent No. 5,358,710 describes a method of suppressing an animals antibody-mediated immune response to

a second antigenic polypeptide by selecting a mammal which is unsensitized to a first antigenic polypeptide, and administering a tolerogenic conjugate of the first antigenic peptide covalently bound to a water soluble polymer. Then an adduct of the first antigenic polypeptide bound to a second antigenic polypeptide is administered. The administration of the tolerogenic conjugate suppresses the capacity of the mammal to mount a humoral antibody response to the first antigenic peptide so that when the first antigenic peptide is conjugated to the second antigenic peptide the anibody mediated immune response to the second antigenic peptide is also suppressed.

Disclosure of the Invention

The goal of this invention is to provide a method for induction of suppression of both humoral (antibody), and cellular (cell mediated) immune responses to an Ag, by the administration of the antigen in the form of a conjugate with mPEG, (i.e. Ag(mPEG) containing an optimal number of mPEG molecules coupled covalently onto the antigen) at any time prior to injection of the Ag by itself or in conjunction with an appropriate adjuvant, so as to induce humoral and cellular responses to the Ag in question. Hence, the immunosuppressive $Ag(mPEG)_n$ conjugate is also referred to as a tolerogenic conjugate.

Part I of the application generally enables methods of suppressing the humoral and cell mediated immune responses. Part II of the application enables specific applications of the above method to gene therapies, organ transplantation and treatment of autoimmune conditions.

In another embodiment, the invention provides a method of obtaining passive transfer of suppression of an immune response comprising treating an animal, preferably a syngeneic animal with Ag(mPEG) conjugate and transferring lymphocytes from said animal to a recipient animal, wherein

said lymphocytes provide suppression of Ag-specific cytotoxic lymphocyte (CTL) activity in said recipient animal.

The invention advantageously provides a method of treating a condition selected from the group consisting of allergies and autoimmune diseases by inducing tolerance to an antigen (Ag) in both humoral and cell mediated immune responses comprising administering an effective amount of Ag(mPEG) conjugate to induce tolerance to an antigen (Ag).

In still another embodiment, the invention provides a method of preventing an immune rejection of organ transplants comprising administering an effective amount of Ag(mPEG) conjugate to induce tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses.

Finally, the invention provides a method of treating organ-specific autoimmune diseases in animal comprising administration of mPEG conjugates of autoantigens selected from the group consisting of collagen-induced arthritis by type II collagen, experimental autoimmune encephalomyelitis by myelin basic protein, and diabetes in NOD mice by insulin to induce tolerance to an antigen (Ag) in both humoral and cell mediated immune responses. The invention provides a method of conducting gene therapy including administering to a mammal an immunosuppressive effective amount of a tolerogenic conjugate consisting of a protein coupled to monomethoxypolyethylene glycol (mPEG) having a molecular weight of about 2,000-10,000 daltons, wherein administration of said tolerogenic conjugate is at least one day prior to administration of a gene therapy vector encoding a gene for a protein, wherein said protein is identical to said protein which is coupled to mPEG, and wherein said method results in the suppression of an immune response and in the development of tolerance to the protein expressed by said gene encoded by said gene therapy vector.

The above and other objects of the invention will become readily apparent to those of skill in the relevant art from the following detailed description and figures, wherein only the preferred embodiments of the invention are shown and described, simply by way of illustration of the best mode of carrying out the invention. As is readily recognized the invention is capable of modifications within the skill of the relevant art without departing from the spirit and scope of the invention.

Abbreviations used herein: Ab, antibody; Ag, antigen, APC, antigen presenting cell; CFA, complete Freund's adjuvant; CTL, cytotoxic T lymphocyte; HIgG, human monoclonal (myeloma) immunoglobulin G; IFN-γ interferon; IL, interleukin, LNL, lymph nodal lymphocyte; MHC, major histocompatibility complex; mPEG, monomethoxypolyethylene glycol; OVA, ovalbumin, PCA, passive cutaneous anaphylaxis; TCR, T cell receptor, Th, helper T cells; Ts suppressor T cell; TsF, Ts factor; f.p., foot pad.

Brief Description of Drawings

FIG. 1 shows that OVA(mPEG) conjugate induces suppression of Ab responses. Groups of C57BL/6 mice were injected i.p. with PBS, 2.5 mg OVA(mPEG) or 1 mg OVA. After one week, mice were primed via the f.p. with 200 μg OVA in CFA and bled two weeks later. Sera from each group were tested individually for OVA-specific IgG (*upper panel*) or IgG isotypes (*lower panel*, at 1:100 dilution) by ELISA and absorbance was read at 405 nm. Normal mouse sera were used as negative control and results are shown as averages of three mice $\pm$ SD.

FIG. 2 shows specificity of suppression of humoral and cell mediated immune responses induced by conjugates of protein Ag and mPEG. C57BL/6 mice were either untreated, or injected i.p. with 2.5 mg OVA(mPEG) or 2.5 mg HIgG(mPEG). After one week, half of each group of mice were primed via

f.p. with 200 μg OVA in CFA and the other half with 200 μg HIgG in CFA. Mice were bled two weeks later and sera (1:50 dilution) from each group were tested individually for IgG specific for OVA (*left panel*) or HIgG (*right panel*) by ELISA, and absorbance was read at 405 nm.

Normal mouse serum was used as negative control and the results are shown averages of three mice ± SD.

FIG. 3 shows that OVA(mPEG) conjugate induces suppression of lymphokine production by helper T cells. C57BL/6 mice were pretreated and primed as described in Figure 1. Two weeks after immunization, draining lymph nodes were harvested and 2 x 10^6 of LNL were incubated at 37°C with 2 x 10^6 of irradiated APC in the presence or absence of 200 μg/ml OVA. As control, LNL were incubated with 200 μg/ml ConA in the absence of APC. Supernatants were harvested 24 h later and tested for lymphokine production using HT-2 cells. Results are shown as averages of triplicates ± SD.

FIG. 4 shows that OVA(mPEG) conjugate induces suppression of lymphokine production by T cells. C57BL/6 mice were pretreated and primed as described in Figure 1. Draining lymph nodes were harvested two weeks after immunization and LNL were incubated with APC in the presence or absence of OVA as described in Figure 2. Supernatants were tested for IL-2, IL-4 and IFN-γ by lymphokine ELISA.

FIG. 5 shows that OVA(mPEG) conjugate inhibits activation of T cells. C57BL/6 mice were primed via f.p. with 200 μg OVA in CFA. Two weeks later, 3 x 10^6/ml of spleen cells were incubated with medium alone, 200 μg/ml OVA or 500 μg/ml OVA(mPEG) conjugate. After 24-h incubation at 37°C, the supernatants were tested for IFN-γ lymphokine ELISA.

FIG. 6 shows that OVA(mPEG) conjugate induces suppression of cytolytic responses. C57BL/6 mice were

injected i.p. with (A) PBS, (B) 2.5 mg OVA(mPEG) or (C) 1 mg native OVA. After one week, mice were primed via f.p. with 200 μg OVA in CFA. Spleens were harvested two weeks later and spleen cells were stimulated with irradiated E.G7-OVA cells. After six days, cytolytic activity of cultured cells was measured using ^{51}Cr-labeled E.G7-OVA or EL4 targets. Results are shown as % specific lysis at various E:T ratios and represent averages of triplicates ± SD.

FIG. 7 shows that immunosuppression could be achieved by transfer of spleen cells of syngeneic mice that had been tolerized by OVA(mPEG). Donor C57BL/6 mice were injected i.p. with (B) PBS, (C) 2.5 mg OVA(mPEG) or (D) 1 mg OVA. One week later, 1 x 10^8 of spleen cells were transferred i.v. to syngeneic naive mice. One day after adoptive transfer, control mice (A) and recipients (B to D) were primed via f.p. with 200 μg OVA in CFA. Two weeks after priming, spleen cells from recipients were stimulated with irradiated E.G7-OVA cells for six days and their cytolytic activity was tested in a 4-h standard ^{51}Cr-release assay as described in Figure 6.

FIGS. 8, 9 and 10 show diagrams illustrating the efficiency of the invention. The percentages in brackets of Figs. 8 and 10 represent the degree of suppression with respect to the control in animals receiving phosphate buffered saline (PBS) in lieu of the conjugates.

FIG. 11 shows mice injected with human insulin substituted with 3 mPEG groups and later challenged with human insulin developed significantly lower levels of antibody than the control group ($p=0.020$) by Student's t-test.

Description of the Invention

Part-I Suppression of Humoral and Cell-Mediated Immune Response

The present invention provides a method for inducing specific suppression to a given antigen (Ag) of both humoral

and cell-mediated immune responses comprising administering an effective amount of a tolerogenic Ag(mPEG) conjugate. In one embodiment the tolerance induced by Ag(mPEG) conjugates is Ag specific and the suppression of the humoral response is induced in an isotype-nonspecific manner. The tolerance is mediated by Ag-specific $CD8^+$ suppressor T (Ts) cells.

The conjugate, in another embodiment, suppresses IL-2 production by lymph node lymphocytes (LNL). In yet another embodiment the conjugate suppresses IL-2, IFN-γ and IL-4 lymphokine production. The method does not skew $CD4^+$ T cells toward Th1 or Th2 phenotype. The Ag(mPEG) conjugate inhibits *in vitro* lymphokine production by in vivo primed $CD4^+$ Th cells. The Ag(mPEG) conjugate advantageously inhibits both arms of cell-mediated immune responses *in vivo*.

The invention also provides a method for obtaining passive transfer of suppression of an immune response comprising treating an animal with Ag(mPEG) conjugate and transferring lymphocytes from said animal to a recipient animal, wherein said lymphocytes provide suppression of Ag-specific cytotoxic lymphocyte (CTL) activity in said recipient animal. In this method the transfer of Ts cells inhibited cytolytic responses in recipients. Also the transfer of splenic Ts cells from an Ag(mPEG)-tolerized animal leads to downregulation of primary IgE and IgG responses in recipient animals.

This ability to suppress the immune response provides a method of treating a condition preferably selected from the group consisting of allergies and autoimmune diseases by inducing tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses comprising administering an effective amount of Ag(mPEG) conjugate to induce tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses.

The ability to suppress the immune response also

provides for a method of preventing an immune rejection of organ transplants, or rejection of DNA transfected cells and their expressed protein product, comprising administering an effective amount of Ag(mPEG) conjugate to induce tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses. In this method antibodies of all IgG subclasses are suppressed. IgG isotopes dependent upon Th1 and Th2 lymphokines are both inhibited by said Ag(mPEG) conjugates. In addition lymphokines produced by $CD4^+$ Th cells are inhibited by said Ag(mPEG) conjugate. These lymphokines are selected from the group consisting of IL-2, IL-4 and IFN-γ.

Finally the invention provides a method of treating organ-specific autoimmune diseases in animal comprising administration of mPEG conjugates of autoantigens selected from the group consisting of collagen-induced arthritis by type II collagen, experimental autoimmune encephalomyelitis by myelin basic protein (58), and diabetes in NOD mice by insulin to induce tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses.

The inventors demonstrated the induction of $CD8^+$ T cells activated by exogenous protein antigens (Ag) in immune regulation. It has been demonstrated that exogenous ovalbumin (OVA) primed murine $CD8^+$ cytotoxic T lymphocytes (CTL) precursors, if administered with complete Freund's adjuvant (CFA), these $CD8^+$ CTL suppressed Ag-specific humoral and cell-mediated immune responses in recipients after transfer. Thus, $CD8^+$ CTL are immunosuppressive and can be accounted as regulatory cells in some situations. The inventors have shown that oral administration of protein Ag induced tolerance of both humoral and cell-mediated immune responses. This tolerance was mediated by Ag-specific $CD8^+$ suppressor T (Ts) cells that were phenotypically distinguished from $CD8^+$ CTL by reactivity with a monoclonal antibody (mAb) specific for the murine Ts cells.

The present inventors have found that mPEG modified ovalbumin (OVA), which served as a model antigen, induced suppression of cell-mediated responses stimulated by OVA in CFA, as measured by the inhibition of secretion of the lymphokines, IL-4 and IFN-γ by $CD4^+$ T cells and inhibition of the development of OVA-specific, $CD8^+$, CTL's. This effect was antigen-specific in that responses to unrelated antigens were not inhibited by mPEG modified OVA (and vice versa). Furthermore, tolerance could be transferred to normal recipients by spleen cells from syngeneic donor mice which had been treated with mPEG modified OVA, i.e. these spleen cell recipients dramatically reduced the priming of CTL's in the cell. These results demonstrate that to prevent cell-mediated, as well as humoral, immune responses are susceptible to antigen-specific immunosuppression by mPEG conjugates of the appropriate antigen. These observations show that this strategy can be used to prevent, or reverse cell-mediated responses in a variety of clinical conditions.

Correction of genetic defects by the transfer of DNA encoding the corresponding non-defective genes is currently the object of intense investigation. One of the limitations of this technology is that the proteins expressed by foreign genes induce antibody responses in recipients deficient of the gene in question. The antibodies bind to the proteins and inhibit their activity by decreasing their effective half-life in circulation. Pretreatment of such recipients with mPEG modified proteins prior to gene therapy would prevent this response. Moreover, it has become clear that proteins encoded by the transferred genes behave as viral genes (i.e. they are first expressed on the cell membrane) and consequently induce cell-mediated immune responses (i.e. generated CTL's are potentially capable of destroying by cytolytic mechanisms, the cells expressing the transgene). These results demonstrate that both antibody and cell-

mediated immune responses to the protein products of the transferred genes is inhibited by pretreatment with mPEG modified proteins. Hence this strategy also extends the usefulness of gene transfer procedures.

In the present invention the inventors investigate the mechanisms of immune regulation by using the tolerogenic conjugates of protein Ag and mPEG. The results show that a single injection of OVA(mPEG) conjugate but not unconjugated OVA suppressed subsequent Ab responses, lymphokine production by $CD4^+$ T cells and inhibited priming of $CD8^+$ CTL precursors to OVA. The tolerance induction by Ag(mPEG) conjugates was Ag specific, and suppression could be transferred by splenic T cells from tolerized mice to syngeneic mice. Thus, the inventors confirmed that Ag(mPEG) conjugates not only suppress Ab (i.e. humoral) responses suppressing different, i.e., Ig classes but also inhibit cell-mediated immune responses *in vivo*.

Covalent coupling of diverse protein antigens and mPEG resulted in Ag(mPEG) conjugates that have been found to be tolerogenic rather than immunogenic (18-22). Administration of Ag(mPEG) conjugates is a well-established method to induce long-lasting tolerance (20,23) and has been used to prevent the induction of Ag specific allergic responses which are mediated by IgE Abs (19,24). Ag(mPEG) conjugates stimulated the induction of Ag-specific, non cytotoxic Ts cells (25,26), which expressed Thy-1 and $CD8^+$ markers (26,27), and reacted with a mAb specific for the activated murine Ts cells (17). Clones of nonhybridized $CD8^+$ Ts cells produced a soluble Ts factor (TsF) (26,27) that was serologically and physicochemically related to the $\alpha\beta$ heterodimer of TCR (28). Functionally, these $CD8^+$ Ts cells and TsF suppressed *in vitro* and *in vivo* Ab production in an Ag-specific and MHC class I-restricted manner (27,30,31). However, TsF had no suppressive effect on fully differentiated B cells or plasma cells but exerted its down

regulating effect on Ag-specific Th cells through interaction with normal $CD8^+$ T cells in the presence of APC and Ag (31,32). Moreover, $CD8^+$ Ts cells produced a Th0-like pattern of cytokines (IL-2, IL-4, IFN-γ, TGF-β, TNF-α and lymphotoxin) that were found not to be directly responsible for the observed downregulation of Ab responses (30).

Using this model system, the inventors investigated the tolerance induction of cell-mediated immune responses by Ag(mPEG) conjugates. Indeed, in addition to inducing suppression of humoral responses, OVA(mPEG) conjugate but not the unmodified OVA, inhibited lymphokine production by $CD4^+$ Th cells and priming of $CD8^+$ CTL precursors upon challenge with OVA in CFA. Moreover, tolerance induced by OVA(mPEG) conjugate was mediated by Ts cells that induced also Ag-specific suppression on transfer to naive syngeneic recipients.

Example 1

Animals. Eight- to 12-week-old female C57BL/6, mice ($H\text{-}2^b$) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and used exclusively in this study. Outbred, 250 to 350 g male Sprague-Dawley or Long Evans hooded rats were obtained from the Central Animal Care Services of the University of Manitoba.

Antigens and tolerogens. Purified chicken egg OVA (grade VI) was purchased from Sigma Chemical Co. (St. Louis, MO). Human monoclonal IgG (HIgG) was isolated from serum of a myeloma patient by ammonium sulfate precipitation and ion exchange chromatography on DEAE (20). mPEG (average M_r = 3200 Da) was obtained from Pharmacia AB (Uppsala, Sweden). The tolerogenic conjugates used in this study, $OVA(mPEG)_{10}$ and $HgG(mPEG)_{25}$, were prepared as described earlier (23,25,33 incorporated by reference herein) and dissolved in phosphate-buffered saline (PBS, pH 7.4). Note that any antigen may be used in conjunction with mPEG having a

molecular weight in the range of about 3000-25,000, preferably 5000-20,000. The subscript n in the formula $Ag(mPEG)_n$ represents the degree of conjugation, i.e. the average number of mPEG molecules coupled per molecule of protein Ag. (Although the example presented herein uses OVA Ag as a model antigen, any antigen to which tolerance is desired can be converted to an immunosuppressive mPEG conjugate and be used according to the method of the present invention.)

In an alternative embodiment the tolerogenic conjugates $OVA(mPEG)_{14}$ and $HIgG(mPEG)_{25}$, were synthesized by a modification of the procedure previously reported (21). Aggregate free protein is concentrated by ultrafiltration iva an Amicon filter to 5-10 mg/ml and then reacted with a large excess, an 8-molar excess of the electrophilically activated mPEG intermediate e.g., mPEG para-nitrophenol carbonate (which reacts with alpha and epsilon amino groups), commercially available from Shearwater Polymers, Huntsville, Alabama, with respect to the total lysine content of the protein. This procedure is applicable to any protein.

By way of example, 47 mg of OVA (1mM), which has 19 lysines per molecule of OVA[1] (i.e., 19 mM of lysines) was reacted with 486.4 mg of activated intermediate {152 (8 x 19) millimoles}. For 150 mg of IgG (1mM), with an average lysine content of 90 per molecule of HIgG, the IgG was reacted with 2.3 grams of mPEG. The protein was dissolved at 10 mg/ml in 0.1 M borate buffer (pH 9.7) and the intermediate was dissolved in a similar volume of double distilled water (DDW). The protein was added quickly with stirring to the intermediate solution. The reaction mixture was then added to a dialysis bag and suspended in 4 liters

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[1] Nisbet, A.D., Saundry, R.H., Moir, J.G., Fothergill, L.A. and Fothergill, J.E. 1981, Eur. J. Biochem. 115:335-345.

of 0.05M Borate buffer (pH 9.7) for 1 hour at room temperature and overnight in the cold. The liquid outside the dialysis bag was constantly stirred and served as a pH-stat. The content of the dialysis bag was applied to a Pharmacia BloPilot gel filtration column (Superdex 60/600) equilibrated with DDW for the isolation of the conjugate, which elutes in the void volume, from the hydrolyzed mPEG and from the para-nitrophenol which elutes with the buffer salts, and to place the conjugate in DDW for lyophilization. The conjugate is stored lyophilized at -20°C.

Tolerance induction and immunization protocol. C57BL/6 mice were pretreated by intraperitoneal (i.p.) injection with 2.5 mg OVA(mPEG) or 1 mg native OVA. The control mice received 0.5 ml PBS in lieu of Ag. The OVA(mPEG) conjugates used in this study contained ~40% (wt/wt) protein, i.e. 2.5 mg OVA(mPEG) conjugates contained about 1 mg native OVA within the complex. Seven days later, mice were immunized in the hindfoot pads (f.p.) with 200 μg of OVA emulsified in CFA containing *M. tuberculosis* H37Ra (Difco Labs, Detroit, MI). Two weeks after immunization, mice were bled and sacrificed. Draining lymph nodes and spleens were harvested for testing lymphokine production and cytolytic activity, respectively (4,13). In some control experiments, mice were pretreated with 2.5 mg HIgG(mPEG) and then immunized with 200 μg HIgG in CFA.

Passive cutaneous anaphylaxis (PCA) assay. The anti-OVA IgE levels in primed sera mice were determined by the PCA in male Sprague-Dawley or Long Evans hooded rats as described elsewhere (34). Rats were anesthetized by injecting 1% Nembutal i.p. and the backs were shaved from shoulder to rear haunches. Volumes of 50 μl of two-fold serial dilutions of individual sera were injected intradermally into the backs of the rats. Rats were challenged 4 h later by an intravenous (i.v.) injection of 1 mg OVA in 1 ml PBS containing 1% Evan's Blue. The PCA titer

was expressed as the reciprocal of the highest dilution of the serum giving a blue area of at least 5 mm in diameter. The Student's *t*-test was used to compare the PCA titer of each group with that of the control. The difference was considered statistically significant only when the *p* value was smaller than 0.01.

Enzyme-linked immunosorbent assay (ELISA). A solid-phase ELISA was used to determine Ag-specific IgG production in primed mice (13). Microliter plates were coated with OVA or HIgG at a concentration of 10 μg/ml in borate-buffered saline (BBS, pH 8.2) overnight at 4°C and then blocked with PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide. The test sera were added in three-fold serial dilutions and incubated at 37°C for 1 h. Plates were washed extensively with PBS between each step. Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Cappel, Durham, NC) was then added and incubated at 37°C for 1 h. For determining OVA-specific IgG isotypes, rabbit anti-mouse IgG heavy chains (γ_1, γ_{2a}, γ_{2b} and γ_3) (Zymed, South San Francisco, CA) and AP-conjugated goat anti-rabbit IgG were used as primary and secondary detecting Ab, respectively, the *p*-nitrophenyl phosphate substrate dissolved in diethanolamine buffer (pH 9.8) was added to each well and absorption was read at 405 nm using an automatic microplate reader (Molecular Devices Corp., Menlo Park, CA).

Lymphokine production. Lymphocytes from draining lymph nodes (LNL) were harvested two weeks after f.p. immunization with OVA in CFA. LNL (2×10^6) were cultured with 2×10^6 of irradiated (2,000 rad) syngeneic splenic APC with or without 200 μg/ml OVA in 1 ml culture medium consisting of RPMI 1640, 1 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol plus antibiotics. As positive control for T cell stimulation, LNL were incubated with 5 μg/ml concanavalin A (Con A) in the absence of APC.

After 24-h incubation at 37°C, supernatants were harvested, frozen and thawed, then added to the 96-well plates containing 5 x 10_3 per well of IL-2-dependent HT-2 cells (35). After another 40-h incubation at 37°C, proliferation of HT-2 cells was determined by a calorimetric assay using tetrazolium salt XTT (Diagnostics Chemicals, Ltd., Oxford, CT) and phenazine methosulfate as described elsewhere (36). Absorbance was read at 450 nm. All assays were performed in triplicate and reported as the mean ± standard deviation (SD). In some cases, 3 x 10^6/ml of spleen cells from mice primed with OVA in CFA two weeks earlier were incubated with medium alone, 200 μg/ml soluble OVA or 500 μg/ml soluble OVA(mPEG) conjugate at 37°C for 24 h. Supernatants were then tested for lymphokine production by lymphokine ELISA.

Lymphokine ELISA. Supernatants of primed LNL cultured with or without OVA were tested for lymphokine pattern by sandwich ELISA (13). Paired mAb (PharMingen, San Diego, CA) were used as capture and detecting Ab, respectively. Microliter plates were coated with capture Ab in BBS overnight at 4°C. After blocking excess protein-binding sites by PBS plus 1% BSA at 22°C for 1 h, serially diluted recombinant cytokines (IL-2, IL-4 or IFN-γ) and supernatants were added and incubated overnight at 4°C. Biotin-conjugated detecting Ab and avidin-horseradish peroxidase conjugate (Vector Labs, Burlingame, CA) were subsequently added and incubated at 22°C for 45 min and 30 min, respectively.

The plates were washed extensively with PBS plus 0.1% Tween 20 between each step. Calorimetric reaction was developed by exposure to the 2,2′-azino-di[3-ethyl-benzathiazoline sulfonate] (ABTS) substrate (Kirkegaard & Perry Labs, Gaithersburg, MD) and absorbance was read at 405 nm. The cytokine concentration ln each supernatant was calculated from the standard curve of each recombinant cytokine.

Cytotoxicity assay. Spleens from C57BL/6 mice were harvested two weeks after immunization with OVA in CFA (4). Single cell suspensions were prepared and erythrocytes were lysed. Mononuclear spleen cells (35×10^6) were stimulated *in vitro* for 6 days with 3×10^5 of irradiated (20,000 rad) syngeneic E.G7-OVA cells according to the method described by Moore et al. (6).

E.G7-OVA is an Ia$^-$ EL-4 (H-2^b) thymoma clone transfected with the chicken OVA cDNA gene (6) (provided by Dr. M. J.Bevan, University of Washington, Seattle, WA). The E.G7-OVA clone provides a model for gene therapy applications of the invention. Cytolytic activity of cultured spleen cells was determined in a standard 4-h ^{51}Cr-release assay (13). Percent of specific lysis was calculated as: (^{51}Cr release by effector cells - spontaneous ^{51}Cr release) / (maximal ^{51}Cr release - spontaneous ^{51}Cr release). Maximal ^{51}Cr release was achieved by adding 1% Triton X-100 to the target cells. Spontaneous ^{51}Cr release in the absence of effector cells was generally <10% of the maximal release in all experiments. All assays were performed in triplicate and reported as the mean $\pm$ SD.

This example shows that mPEG conjugates inhibit specific cytotoxic T cells (CTL). The above model system for gene therapy utilized tumor cells transfected with the cDNA for the ovalbumin gene (E.G7-OVA). OVA-specific CTL were engendered by priming mice with OVA in Freund's complete adjuvant. The resulting CTL lysed tumor cells that had been transfected with the OVA gene, but not cells transfected with the insulin gene. It was advantageously found that pretreatment of mice with OVA(mPEG)$_{10}$ inhibited the priming of CTL by OVA in CFA, as well as OVA-specific Ab responses, whereas unmodified OVA had no effect. Thus the gene therapy model showed suppression of the immune response to OVA protein expressed from the chicken OVA cDNA gene.

Transfer of spleen cells from tolerized donor mice. For cell transfer, donor mice were injected i.p. with PBS, 2.5 mg OVA(mPEG) or 1 mg native OVA and sacrificed 7 days later. Spleens were harvested and 1 x 10^8 of spleen cells were transferred into syngeneic naive mice by i.v. injection (5). One day after transfer, recipients were primed f.p. with 200 μg OVA in CFA. Spleen cells from recipients were harvested two weeks later and stimulated *in vitro* with irradiated E.G7-OVA cells as described above.

Discussion

Suppression of humoral responses by OVA(mPEG) conjugate, but not by native OVA. Although inactivation of sensitized mast cells and prevention of systemic anaphylaxis by Ag(mPEG) conjugates have been reported (19,24), the effect of mPEG-AG conjugates on cell-mediated immune responses had not been studied.

In three separate experiments, the anti-OVA IgE responses of all OVA(mPEG)-pretreated mice were suppressed by more than 80% in comparison with that of control (Table 1). Although there was a great variation of anti-OVA IgE titers in mice pretreated with native OVA (Table 1), suppression of IgE responses in these mice was marginal (<28%) as compared to control mice. Thus, these data confirmed that Ag(mPEG) conjugates were more tolerogenic and that the unmodified protein Ag exerted deviation of the immune response to other Ig classes.

To determine whether OVA(mPEG) conjugates also inhibited IgG responses in mice immunized with OVA in CFA, sera from pretreated and subsequent immunized mice were tested for OVA-specific IgG production by ELISA. As illustrated in the upper panel of Fig. 1, OVA(mPEG) conjugate profoundly inhibited IgG responses to OVA, whereas native OVA given i.p. did not suppress IgG responses. This was consistent with our earlier observation that Ab

responses were inhibited by i.v. but not by i.p. injections of soluble Ag prior immunization with the same Ag in adjuvant (37). Next, it was questioned whether OVA(mPEG) conjugates inhibited all IgG isotypes stimulated by OVA in CFA or preferentially, suppressed some, while augmenting other IgG isotypes. As shown in the lower panel of Fig. 1, all IgG subclasses including predominant IgG1 and IgG2b isotypes were inhibited by OVA(mPEG) treatment. Hence, it was concluded that, consistant with Ts cell-mediated suppression (30), OVA(mPEG) conjugate induced tolerance of humoral responses in an antigen-specific and isotype-unspecific manner.

The specificity of tolerance induced by Ag(mPEG) conjugates was then verified by pretreating mice with OVA(mPEG) or HIgG(mPEG). One half of the mice from each group were then immunized with OVA in CFA and the other half with HIgG in CFA. Sera from tolerized mice showed that OVA(mPEG) inhibited only OVA-specific but not HIgG-specific IgG responses, whereas HIgG(mPEG) suppressed anti-HIgG but not anti-OVA IgG responses (Fig. 2). Thus, tolerance induced by Ag(mPEG) conjugates is Ag specific (30).

Suppression of lymphokine production by OVA(mPEG) conjugate but not by native OVA. The tolerogenic effect of OVA(mPEG) conjugate on OVA-specific cell-mediated responses was determined next. Mice were pretreated i.p. with OVA(mPEG) or OVA and then primed f.p. with OVA in CFA as described above. When cultured with syngeneic APC and soluble OVA, LNL from untreated control mice primed with OVA in CFA produced IL-2 that supported the growth of IL-2-dependent HT-2 cells (Fig. 3). Pretreatment with OVA(mPEG) conjugate but not native OVA profoundly suppressed IL-2 production by LNL (Fig. 3). However, LNL from all three groups responded equally well to the mitogen Con A (Fig. 3), indicating that Ag(mPEG) conjugates are not non-pecific toxic to T cells nor induce a deletion of T cells.

To further determine if OVA(mPEG) conjugate might induce an immune deviation by altering pattern of lymphokines produced by $CD4^+$ Th cells, lymphokine production by LNL upon stimulation with OVA was tested. LNL from control mice produced IL-2 and IFN-γ but little IL-4 (Fig. 4). Pretreating mice with OVA(mPEG) conjugate suppressed production of all three lymphokines rather than altering one or another, suggesting that OVA(mPEG) treatment did not skew up $CD4^+$ T cells toward Th1- or Th2-like phenotype at this stage. It is noteworthy that, in contrast to a recent report from Degermann et al, that administration of soluble protein could divert a clonal population of TCR transgenic T cells from Th1 toward Th2-type responses (38), there was not observed an inhibition of $CD4^+$ T cell responses or a deviation of lymphokine pattern by pretreating normal mice with soluble OVA prior to priming with OVA in CFA, suggesting that soluble OVA may have no obvious effect on priming of naive T cells.

Next, it was questioned whether Ag(mPEG) conjugates had downregulating effect on primed $CD4^+$ Th cells. Spleen cells from mice primed with OVA in CFA were cultured with soluble OVA, OVA(mPEG) conjugate or medium alone as control. Supernatants were harvested after 24-h and tested for lymphokine production. Interestingly, OVA(mPEG) conjugate also inhibited *in vitro* lymphokine production by *in vivo* primed $CD4^+$ Th cells (Fig. 5). These results indicate that Ag(mPEG) conjugates prevent not only *in vivo* priming of T cells, but also inhibit *in vitro* activation of primed T cells.

TABLE 1

OVA(mPEG) Conjugate Induces Suppression of IgE Responses

Expt.	i.p. Treatment[a]				
	PBS	OVA(mPEG)		OVA	
	IgE Titer[b]	IgE Titer	%Suppression[c]	IgE Titer	%Suppression
1	320	<40		80	
	160	<40	>81*	640	0
	160	<40		160	
2	>80	<10		>80	
	>80	<10	>88*	>80	<25
	>80	<10		>20	
3	320	<20		40	
	320	<20	>92*	160	<28
	80	<20		320	

[a] Groups of three C57BL/6 mice were injected i.p. with PBS, 2.5 mg OVA(mPEG) conjugate or 1 mg native OVA. One week later, mice were immunized f.p. with 200 μg OVA in CFA.

[b] Mice were bled two weeks after f.p. immunization and individual sera were tested for OVA-specific IgE by PCA in rats. IgE titer was expressed as the reciprocal of the highest serum dilution giving a blue area greater than 5 mm.

[c] % suppression was calculated as: 100 x (mean IgE titer of control group - mean IgE titer of test group)/(mean IgE titer of control group).

* Statistically significant difference between test group vs. control group ($p < 0.01$).

Suppression of cytolytic responses by OVA(mPEG) conjugate but not by native OVA. To test if Ag(mPEG) also induced suppression of cytolytic responses, C57BL/10 mice were pretreated with PBS, OVA(mPEG) conjugate, or native OVA and primed with OVA in CFA. Spleen cells from these primed

mice were then stimulated *in vitro* with irradiated E.G7-OVA cells to induce CTL activity (4).

Control mice primed with OVA in CFA developed OVA-specific T cells that lysed E.G7-OVA targets (Fig. 6A). Pretreatment with OVA(mPEG) conjugate (Fig. 6B), but not with native OVA (Fig. 6C), inhibited priming of OVA-specific CTL and prevented development of cytolytic activity in mice that were subsequently challenged with OVA in CFA. Thus, it was concluded that Ag(mPEG) conjugates inhibited both arms of cell-mediated immune responses in vivo, that is both humoral and cell mediated responses.

Transfer of tolerance by splenic T cells induced by OVA(mPEG) conjugate. It had been shown in previous studies that transfer of splenic T cells from mice tolerized by HIgG(mPEG) or OVA(mPEG) conjugates into syngeneic recipients led to significant long-lasting suppression of Ab responses, as demonstrated by the specific immunological refractoriness to subsequent immunization of aggregated Ag (25,30). To determine whether spleen cells from mice pretreated with OVA(mPEG) conjugate also transferred tolerance and inhibited CTL responses in recipients, transfer experiments were performed. Donor C57BL/6 mice were injected i.p. with 2.5 mg OVA(mPEG) conjugate, 1 mg native OVA, or PBS as control. Spleen cells were harvested and transferred into naive syngeneic mice. Recipient mice were primed f.p. with OVA in CFA one day later and sacrificed after two weeks.

Spleen cells from recipients were then stimulated *in vitro* with irradiated E.G7-OVA cells as described above. As illustrated in Fig. 7, transfer of spleen cells from PBS-treated control mice (Fig. 6B) or from OVA-treated mice (Fig. 6D) had no obvious suppressive or enhancing effect on the CTL responses in recipients, in relation to the CTL activity of primed mice that had not received any donor cells (Fig. 6A). In contrast, transfer of spleen cells from mice that had been treated with OVA(mPEG) conjugate resulted

in a profound suppression of OVA-specific CTL activity (Fig. 6C). These data suggested that inhibition of cytolytic responses in recipients was mediated by Ts cells rather than a result of diluting CTL precursors by transferred cells, and the magnitude of the suppression effect was correlated to the number of donor cells transferred to the recipients (data not shown).

The possibility of selectively downregulating the host's immune responses to a given Ag represents one of the most formidable challenges of modern immunology in relation to the development of new therapeutics for allergies, autoimmune diseases, and prevention of immune rejection of organ transplants. Similar considerations apply to an increasing number of promising therapeutic modalities for a broad spectrum of diseases, which involve the use of chemical or biologically active agents potentially capable of modulating immune responses, provided they were not allergic or immunogenic. Among these agents, the copolymeric conjugates of protein Ag and mPEG were found not only essentially non-immunogenic but also immunosuppressive and tolerogenic. This observation has been evidenced by the fact that administration of mPEG conjugates of a highly immunogenic protein Ag to rodents resulted in the suppression of their capacity to mount IgE responses to the unmodified Ag and these animals were also systemically anergic to the injection of the same Ag (39,40).

Furthermore, experiments have shown that transfer of spleen cells from mice tolerized by Ag(mPEG) conjugates into naive syngeneic recipients led to significant suppression of IgE or IgG responses in the latter to subsequent injections of the corresponding Ag (25,30,39). The results show that the specific immunosuppression induced by tolerogenic Ag(mPEG) conjugates involves the activation of Ag-specific Ts cells.

The tolerogenic Ag(mPEG) conjugates induced a profound suppression of immune responses in animal models (40). Results from this study have demonstrated that OVA(mPEG) conjugate induced tolerance in both OVA specific humoral and cell-mediated immune responses. Ab of all IgG subclasses were suppressed by pretreating mice with OVA(mPEG) conjugate but not by the native unmodified OVA, suggesting that IgG isotypes dependent upon Th1 and Th2 lymphokines were inhibited by Ag(mPEG) conjugates. This finding was confirmed by the observations that all lymphokines produced by $CD4^+$ Th cells, such as IL-2, IL-4 or IFN-γ, were inhibited by OVA(mPEG) treatment. Hence, unlike oral tolerance where a Th2 response was preferentially induced by oral Ag (41), tolerance induced by Ag(mPEG) conjugates may not reflect an immune deviation by promoting one Th cell subset while suppressing another.

Moreover, pretreatment with OVA(mPEG) conjugate also inhibited subsequent priming of $CD8^+$ CTL precursors and suppressed their cytolytic activity. This tolerance of cell-mediated responses was mainly attributed to the activation of Ag-specific Ts cells that transferred the tolerance to naive syngeneic recipients. Thus, these data evidence that transfer of splenic Ts cells from Ag(mPEG)-tolerized mice led to downregulation of primary IgE and IgG responses in recipients (26,27).

Earlier studies have revealed that Ts cells derived from mice treated with OVA(mPEG) or HIgG(mPEG) conjugates were $Thy\text{-}1^+$, $CD3^+$, $CD4^-$, $CD5^+$, $CD8^+$ and expressed the $\alpha\beta$ heterodimer of TCR (26,27). Nonhybridized $CD8^+$ Ts clones were phenotypically distinguishable from the $CD8^+$ CTL by their expression of the carbohydrate epitope detected with the mAb 984D4.6.5 (17), which is expressed by activated Ts cells but not their precursors, CTL precursors, or activated CTL effectors (42). Moreover, these Ts cells differed from CTL in that they were not cytotoxic and produced Ag-specific

TsF, which suppressed *in vitro* Ab formation (27). Thus, in this tolerance model, $CD8^+$ T cells with cytolytic activity did not account for the biological activity of $CD8^+$ Ts cells induced by Ag(mPEG) conjugates.

The relationship between $CD8^+$ CTL and non-cytolytic, $CD8^+$ Ts cells is currently not clear. They could both be the progeny of a $CD8^+$ T cell precursor with the potential of developing into either subset as demonstrated in the paradigm for development of Th1 and Th2 cells from $CD4^+$ Th cell precursors (43). This idea is supported by the observation that subsets of $CD8^+$ T cells expressing different patterns of lymphokines can be derived from $CD8^+$ T cells obtained from TCR transgenic mice (44). Alternatively, $CD8^+$ CTL and $CD8^+$ Ts cells could be derived from separate precursors having different functions. Regardless of which model proves to be correct, it is clear that once activated, $CD8^+$ Ts cells inhibit both humoral and cell-mediated immune responses. The mechanisms by which such non cytolytic $CD8^+$ Ts cells downregulate immune responses are not entirely clear. However, the soluble TsF secreted by these $CD8^+$ Ts cells might be a plausible explanation for the observed tolerance (26).

Numerous studies have shown that treatment of organ specific autoimmune diseases in animal models could be achieved by oral administration of respective autoantigens {reviewed in (41)}, such as collagen-induced arthritis by type II collagen (45,46), experimental autoimmune encephalomyelitis by myelin basic protein (47,48), experimental autoimmune uveoretinitis by S-antigen (49) or diabetes in NOD mice by insulin (50). However, Heath's group has recently reported that under certain circumstances, feeding mice with autoantigens can cause rather than prevent autoimmune diseases (51,52). Thus, the present inventors' data indicated that conjugates of autoantigens and mPEG could bypass this effect of Ag

administered orally and provide an alternative means to prevent cell-mediated autoimmunity.

Example 2

Human insulin is a protein that has been considered for gene therapy as a treatment for patients with type I diabetes. Human insulin was conjugated with one, two or three mPEG groups/molecule. Three BALB/c mice per group were treated with phosphate buffered saline (PBS) or 20 micromoles human insulin conjugates in PBS intraperitoneally on days -14 and -7. All mice were challenged with 20 microM unmodified human insulin in complete Freund's adjuvant subcutaneously on day 0. Mice were bled and serum tested for insulin-specific antibody by ELISA. Despite the number of mice in the experiment, human insulin substituted with 3 mPEG groups developed significantly lower levels of antibody than the control group ($p=0.020$) by Student's t-test.

The above example confirms the ability to induce tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses by administering an effective amount of an Ag conjugated with monomethoxypolyethylene glycol (mPEG) conjugate.

Part II - Gene Therapy

The ability to suppress the humoral and cell mediated immune responses has several important applications, particularly in the areas of treating allergies, autoimmune diseases, preventing immune rejection in organ transplants and preventing rejection of genetically engineered cells in gene therapy.

Foreign proteins or DNA, such as genetic material or vectors for gene therapy, or their derivatives, have therapeutic properties and are administered to patients suffering from certain diseases. However, the immunogenicity of the said foreign proteins, nucleotides,

DNA or vectors, or of their derivatives, may vitiate the treatment and hence this invention provides an improved method for the treatment of such diseases.

Gene therapy is the insertion of a functioning gene into the cells of a patient (i) to correct an inborn error of metabolism (i.e., genetic abnormality or birth defect resulting in the deficiency of the patient with respect to one or more essential proteins such as enzymes or hormones), or (ii) to provide a new function in a cell Culver, K.W., "Gene Therapy", 1994, p. xii, Mary Ann Liebert, Inc., Publishers, New York, NY).

When the host is totally deficient of the inserted gene from birth, the new protein expressed by this gene --when the latter is inserted into the appropriate cell of an adult host-- would induce in the host an immune response against itself. Hence, (i) the host would produce antibodies or cytotoxic cells to the "new" protein, and (ii) this immune response would not only combine and neutralize and thus inactivate the function of the "new" protein, but may also lead to untoward therapeutic complications due to formation of immune complexes. It is, therefore, not surprising that gene therapy has proven successful in adenosine deaminase (ADA) deficiency, i.e., in children deficient of ADA from birth, which is manifested by the absence of functional T lymphocytes and consequently to the severe combined immunodeficiency (SCID) syndrome.

The reported success of gene therapy in young children deficient of ADA from birth is related to the immunodeficient status of the child, as no immune response can be generated against the foreign therapeutic genetic material. As a corollary, gene therapy would be successful if it is instituted from birth, when it is relatively easy to induce immunological tolerance to a foreign immunogenic material.

This invention provides a method for overcoming this inherent complication due to the immunogenic capacity of the

expressed protein, and is therefore considered to represent a novel and an essential improvement for the treatment of such diseases.

As background to the invention, Bitoh, S., Takata, M., Maiti, P.K., Holford-Stevens, V., Kierek-Jaszczuk, D. and Sehon, A.H., disclose that "Antigen-specific suppressor factors of noncytotoxic $CD8^+$ suppressor T cells downregulate antibody responses also to unrelated antigens when the latter are presented as covalently linked adducts with the specific antigen." Cell. Immunol. 150:168-193, 1993.

Bitoh, S., Lang, G.M., Kierek-Jaszczuk, D., Fujimoto, S. and Sehon, A.H., disclose "Specific immunosuppression of human anti-murine antibody (MAMA) responses in hu-PBL-SCID mice." Hum. Antibod. Hybridomas 4:144-151, 1993. Bitoh, S., Lang, G.M. and Sehon, A.H., disclose the Suppression of human anti-mouse idiotypic antibody responses in hu-PBL-SCID mice." Hum. Antibod. Hybridomas 4:144-151, 1993. Dreborg, S. and Akerblom, E., disclose the safety in humans of "Immunotherapy with monomethoxypolyethylene glycol modified allergens." In: S.D. Bruck (Ed.), CRC Crit. Rev. Ther. Drug Carrier Syst. 6:315-363, (1990).

Generally the term "antigen" refers to a substance capable of eliciting an immune response and ordinarily this is also the substance used for detection of the corresponding antibodies by one of the many *in vitro* and *in vivo* immunological procedures available for the demonstration of antigen-antibody interactions. Similarly, the term allergen is used to denote an antigen having the capacity to induce and combine with reaginic (i.e., IgE) antibodies which are responsible for common allergies. The latter definition does not exclude the possibility that allergens may also induce reaginic antibodies, which may include immunoglobulins of classes other than IgE.

As used herein, the term "antigenicity" is defined as the ability of an antigen (immunogenic material) or allergen

to combine *in vivo* and *in vitro* with the corresponding antibodies; the term "allergenicity" or skin activity is defined as the ability of an allergen to combine in vivo with homologous reaginic antibodies hereby triggering systemic anaphylaxis or local skin reactions, the latter reactions being the result of direct skin tests or of passive cutaneous anaphylactic (PCA) reactions; and the term immunogenicity in a general sense is the capacity of an antigen or allergen, or of their derivatives produced *in vitro* or processed *in vivo*, to induce the corresponding specific antibody response.

In relation to this invention, tolerogens are defined as immunosuppressive covalent conjugates consisting of an antigenic material (immunogenic proteins, such as the expressed protein products of gene therapy vectors, etc.) and a water-soluble polymer (see e.g. Sehon, A.H., In "Progress in Allergy" (K. Ishizaka, ea.) Vol. 32 (1982) pp. 161-202, Karger, Basel; and U.S. patent No. 4,261,973).

In the present context, the term "tolerogen" refers to a conjugate consisting of an immunogenic material (protein or polynucleotide) and a nonimmunogenic conjugate, said tolerogen being immunosuppressive in an immunologically specific manner with respect to the antigen which is incorporated into the tolerogenic conjugate irrespective of the immunoglobulin class which is downregulated. Furthermore, the tolerogen may comprise a conjugate of an essentially nonimmunogenic polymer and an immunogenic biologically active product or derivative of the genetic material used for gene therapy.

The therapeutic administration of foreign immunogenic material induces an immune response leading to the formation of antibodies of different immunoglobulin classes. Hence, on repeated administration, the material may form complexes *in vivo* with such antibodies leading to a poor therapeutic effect by virtue of its being sequestered and neutralized by

the antibodies, or to anaphylactic reactions by combination with reaginic antibodies, or to other untoward conditions, i.e. immune complex diseases due to the deposition of antibody-antigen complexes in vital tissues and organs. Wilkinson et al. "Tolerogenic polyethylene glycol derivatives of xenogenic monoclonal immunoglobulins, Immunology Letters, Vol. 15 (1987) pp. 17-22, disclose the administration time of a tolerogenic conjugate to a non-sensitized individual at least one day prior to challenge with an antigen, and optionally about 6 or 7 days.

The present invention overcomes deficiencies of the prior art by providing methods of inhibiting humoral and cell mediated immune responses. The invention makes possible the administration of gene therapy, which involves the generation of immunogenic material in a patient deficient of the corresponding gene, possible and effective.

Gene therapy procedures as currently practiced involve the administration by itself of a foreign genetic material, or of its biologically active products, and do have certain disadvantages and limitations which are primarily due to their potential immunogenicity in the host deficient of the corresponding gene.

This aspect of the present invention aims at overcoming the above mentioned complications by suppressing the production of antibodies to the foreign therapeutic genetic material and of its expression products, and of thus ensuring the efficacy of gene therapy by the prior administration of imunosuppressive doses of tolerogenic conjugates consisting of therapeutically active and potentially immunogenic materials coupled to nonimmunogenic polymers, thus overcoming or minimizing the risk of inducing anaphylactic reactions or immune complex diseases.

Thus, the invention aims at suppressing substantially an immune response to the protein resulting as a consequence of successful gene therapy, which response would undermine

the therapeutic efficacy of a biologically active genetic material and which may also cause untoward physiological reactions (e.g. anaphylaxis and/or immune complex diseases).

The invention provides a method for conducting gene therapy comprising administration to a mammal of an immunosuppressing effective amount of a tolerogenic conjugate comprising the genetic material and/or its expression product (i.e., the protein of which the patient is deficient) and monomethoxypolyethylene glycol having a molecular weight of about 2,500-10,000 daltons, the above administration being at least one day prior to administration of the therapeutic genetic material for gene therapy, wherein said method results in the specific suppression of the immune response and the active development of specific tolerance to said therapeutic genetic material and/or its expression product(s). Alternatively, multiple copies of a single gene may be insertedinto a gene therapy vector.

In a preferred embodiment the therapeutic genetic material is selected from nucleotides, DNA, RNA, mRNA, which may or may not be attached to or delivered by appropriate vectors for expression of the required therapeutic protein.

In a more preferred embodiment of gene therapy gene delivery vectors may include Moloney murine leukemia virus vectors, adenovirus vectors with tissue specific promotors, herpes simplex vectors, vaccinia vectors, artificial chromosomes, receptor mediated gene delivery vectors, and mixtures of the above vectors.

In an alternative embodiment of the invention which overcomes the immunogenicity of the gene therapy protein, an mPEG conjugate corresponding to a gene therapy protein, is administered prior to the administration of the gene therapy vector encoding a gene for a therapeutic protein.

In still another embodiment of the invention which overcomes the immunogenicity of the gene therapy vector, an

mPEG conjugate corresponding to a vector protein, is administered prior to the administration of the gene therapy vector. In a preferred embodiment, mPEG conjugates of both the vector protein and gene therapy protein are administered prior to conducting gene therapy with a gene therapy vector encoding a gene for a therapeutic protein. The the vector protein and gene therapy protein mPEG may be conjugated together as a hybrid and administered prior to conducting gene therapy with a gene therapy vector encoding a gene for a therapeutic protein.

The objectives of the present discovery are accomplished by a method, wherein an immunosuppressively effective amount of a tolerogen incorporating a foreign genetic material or its active derivative(s) is administered to the mammal prior to the administration of the foreign genetic material or its biologically active derivative(s). The tolerogenic conjugate is preferably administered to individuals who have not received a prior treatment with the foreign genetic material or its product, i.e. to unsensitized individuals.

The invention provides improved methods for gene therapy of different human diseases which can be ameliorated or eliminated by the administration of the appropriate genetic materials, etc. or their therapeutic derivatives, of which the patient is deficient.

The tolerogenic conjugates may be synthesized by covalent or noncovalent attachment of nonimmunogenic polymers to natural or synthetic biologically active proteins such as for example (i) murine or rat monoclonal antibodies to human T-cells which have been used to suppress transplant rejection (Colvin, R.B. et al.; Fed. Proc. 41 (1982) p. 363, Abstr. 554) or as "miracle bullets" for the destruction of tumors (Froese, G. et al.; Immunology 45 (1982) p. 303-12, and Immunological Reviews 62 (1982), Ed. G. Moller, Munksgaard, Copenhagen), (ii) enzymes, such as

superoxide dismutase (Kelly, K. et al.; Cdn. J. of Physiol. Pharmacol., 609 (1982) p. 1374-81) or L-asparaginase (Uren, J.r. et al.: Canc. Research 39 1979) p. 1927-33), or (iii) natural or synthetic hormones.

In the preferred mode of the invention, the tolerogen is a covalent conjugate between monomethoxypolyethylene glycol (mPEG) with molecular weight in the range of 2,500-10,000 daltons and a foreign protein such as ovalbumin (OVA or OA), which served as a model protein.

According to this modality, tolerogens of appropriate composition (i.e. consisting of the genetic material or its expression product and an optimal number of mPEG chains attached to it covalently) substantially suppress the formation of antibodies of different classes (e.g. IgE and IgG) which are directed specifically against the genetic material *per se* and/or against its expression product(s). The latter case is exemplified by OVA.

Animal model

The acceptability of the mouse as an experimental model for correlation to human utility in the present experiments is evidenced by Dreborg et al. "Immunotherapy with Monomethoxypolyethylene Glycol Modified Allergens", page 325, which indicates that similar results were achieved in humans and mice and thus confirms mice are an acceptable experimental model for evaluation of mPEG modified allergens. See also Antibodies: A Laboratory Manual, Cold Spring Harbor Press, 1988, p. 93, which indicates that laboratory mice are an acceptable experimental animal model for examining the immune response, and that mice, in particular, possess appropriate characteristics for studies of the genetics of the immune response.

The Tolerogen Employed

As water-soluble polymers to be used for the

preparation of a tolerogen, polyethylene glycols, having molecular weights in the range of 2,000 to 35,000, preferably 4,000 to 20,000, have proved to be effective. Polyethylene glycols in this context also include physiologically acceptable derivatives thereof, such as mono-alkyl ethers, preferably the monomethyl ether, whereby the remaining single terminal hydroxyl groups of the molecules are conveniently used for coupling to the protein. Also other water-soluble polymers (macromolecules) may be used, such as polyvinylalcohols, polyvinyl pyrrolidones, polyacrylamides and homo- as well as hetero-polymers of amino acids, polysaccharides (e.g. pullulan, inulin, dextran and carboxymethyl cellulose) or physiologically acceptable derivatives of these polymers.

For the covalent coupling of such polymers to the genetic material or its antigenic expression molecules, chemical methods normally used for coupling of biologically active materials to polymers may be used. Such methods include coupling by means of mixed anhydride, cyanuric chloride, isothiocyanate, reaction between SH derivatives and CH_2I derivatives of the reacting molecules. However, it is obvious to the workers skilled in the art that other appropriate chemical methods may be used to lead to the production of conjugates of desired compositions.

The coupling reaction is made between active groups in the antigen molecules and in the polymer molecules. If necessary such groups may have to be introduced into said molecules before the coupling reaction. Such active groups are for example -NH2, -NCS, -SH, -OH, $-CH_2I$ and -COOH and they may be introduced according to well-known methods, if not already present in the molecules used for the production of tolerogenic conjugates.

In order to minimize the liberation in vivo of the immunogenic and/or allergenic constituent(s) of the tolerogenic conjugates and to maximize their effectiveness

at a low dose, it is desirable that the covalent link between the water-soluble polymer and protein or its active derivative(s) should be as stable as possible under physiological conditions.

The coupling of the polymer onto the antigenic or genetic material must, as mentioned above, have been carried out to such an extent that the conjugate is rendered tolerogenic, as well as substantially non allergenic and substantially non-immunogenic. In other words the tolerogens must retain a certain number of epitopes of the unmodified antigen, as long as their immunogenicity has been decreased so that they do not induce the formation of antibodies which may cause unacceptable adverse reactions.

To achieve tolerogenicity, the degree of substitution, (also referred to as the degree of conjugation, which is defined as the number of polymer molecules coupled per antigen molecule), varies from one antigen molecule to another depending on the nature and size of the antigen and on the polymer and its molecular eight.

Therefore, for the synthesis of a tolerogenic conjugate of a given antigen it is essential to synthesize a series of conjugates with different degrees of substitution and then establish the special range wherein the above mentioned requirements are fulfilled.

Too low a degree of substitution may result in conjugates still endowed with allergenic and immunogenic properties, and too high a degree of substitution may result in conjugates which are not tolerogenic. One of skill in the relevant art will be able to optimize the degree of substitution using the disclosure examples. The optional substitution range is one in which tolerogenicity is achieved.

One of skill in the art can perform the steps outlined herein and arrive at the appropriate degree of coupling of the nonimmunogenic polymer onto any antigenic protein so as

to achieve immunosuppressive properties.

In view of the finely tuned homeostatic balance of the immune response, which may be easily perturbed either upwards or downwards by the administration of a given antigen depending on its dose, state of aggregation and route of administration, as well as the presence or absence of adjuvants, it is critical when practicing the invention for treatment of appropriate disease conditions, that the tolerogenic conjugates be administered in such a manner as to lead to the downregulation of the immune response with respect to one or more classes of immunoglobulins directed against the unconjugated biologically active product of the genetic material.

Hence, in practicing this invention for treatment of appropriate diseases, the tolerogenic conjugates are to be injected in absence of adjuvants since the adjuvants may counteract their suppressogenic effects. However, the inclusion of adjuvants along with the unconjugated immunogenic material in the examples given below was justified so as to stimulate in experimental animals the enhanced production of antibodies in a relatively short time and to thus test under more stringent conditions the capacity of the tolerogenic conjugates to suppress the immune response in these animals even under these extreme conditions which are particularly favorable for enhancing the immune response.

The terms proteins and polypeptides are used synonymously, herein. In the present context the term "foreign genetic material" refers to a nucleotide, DNA, RNA, mRNA, plasmid, which are used as carriers of the gene and/or the gene itself responsible for the expression of the appropriate protein or protein derivative (fragments included), which are substantially immunogenic in the animal to be treated.

According to one aspect of the invention the genetic

material should be therapeutically effective. Many such proteins, vectors, DNA are known per se (Culver, K.W., "Gene Therapy", 1994, p. xii, Mary Ann Liebert, Inc., Publishers, New York, NY, incorporated herein by reference in its entirety).

For the purposes of example only, vectors may be selected from the group consisting of Moloney murine leukemia virus vectors, adenovirus vectors with tissue specific promotors, herpes simplex vectors, vaccinia vectors, artificial chromosomes, receptor mediated gene delivery, and mixtures of the above vectors. Gene therapy vectors are commercially available from different laboratories such as Chiron, Inc., Emeryville, California; Genetic Therapy, Inc., Gaithersburg, Maryland; Genzyme, Cambridge, Massachusetts; Somatx, Almeda, California; Targeted Genetics, Seattle, Washington; viagene and Vical, San Diego, California.

The effective doses (amounts) and formulations commonly used in gene therapy are also known and may be applied to the present invention, although the invention may alternatively employ reduced or increased doses. In principle, both the biologically active foreign genetic material or its derivatives, as well as the corresponding tolerogenic conjugates, may be administered parenterally in a soluble form in isotonic solution and after removal of aggregates by centrifugation.

Time Intervals for Administration

For the induction of immunological tolerance to a given protein the protocol followed according to the invention comprises the administration initially of an immunosuppressive effective dose (amount) of tolerogen, which is given prior to the administration of the gene which encodes a therapeutically active protein or its product. If necessary, this dose may be portioned and given on repeated

occasions. The immunosuppressive dose which is given may vary from tolerogen to tolerogen, but it has to be administered prior to the entry of the protein into the host's system.

In accordance with principles outlined in the examples, the practitioner skilled in the art can determine the variables such as dose of tolerogen and the minimum interval of time between its administration and the appearance of the immunogenic protein in the host's system. See, for example, references discussed in background of the invention. However, gene therapy, resulting in the production of a "new protein in the protein-deficient patient, should be preceded by administration of the specific tolerogenic conjugate, i.e., the conjugate comprising the same protein and capable of suppressing selectively the immune response of the host with respect to the protein in question.

The tolerogenic conjugate is administered prior to the administration of the gene which expresses the foreign protein. A time period of at least one day prior to the administration of the foreign genetic material is preferred. In a more preferred embodiment, the tolerogenic conjugate is administered at least about six days prior to administration of the foreign genetic material.

The immunosuppressive dose refers to the amount of tolerogen required to substantially reduce the immune response of the patient to the protein or to its derivative(s) which will be produced as a result of the gene therapy. According to one mode of the invention, further doses of the tolerogen may be given in conjunction with the protein or its derivative(s), i.e. after the primary administration of the tolerogen.

This mode may represent one way of sustaining the suppression of the humoral and cellular immune responses and offers a more efficient therapeutic regimen for the disease condition for which the treatment has been designed.

The invention will now be illustrated by some non-limiting, representative examples wherein OVA and its tolerogenic mPEG derivatives have been applied as model substances to confirm the usefulness of the proposed immunosuppressive treatment of a well-established animal model commonly utilized in the field of immunology.

The conjugates are designated as OVA-$(mPEG)_n$ where n represents the average degree of conjugation. Figures 8, 9 and 10 show diagrams illustrating the efficiency of the invention. The percentages in brackets of Figs. 1 and 3 represent the degree of suppression with respect to the minimal immune response in animals receiving phosphate buffered saline (PBS) in lieu of the conjugates.

Example 3

Preparation of OVA-mPEG conjugates having different degrees of substitution[2]

The conjugates used in the experiments given below have been prepared by coupling mPEG molecules to OVA essentially according to the procedure described by Abuchowski et al., *J. Biol. Chem.*, Vol. 252, p. 3518 (1977) utilizing cyanuric chloride as one of the possible coupling agents. To begin with, in the experiment described the "active intermediate" consisting of an mPEG molecule attached to cyanuric chloride was prepared. It was found that the most important condition of this reaction was that all reagents be completely anhydrous and that the reaction mixture be protected from atmospheric moisture because of its high susceptibility to hydrolysis.

Among various methods used for the synthesis of the "active intermediate", the example given below illustrates the general procedure. (See also Jackson, C. J.C.,

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[2] Note this is an older method of preparing mPEG conjugates, newer method are set for in the specification at page 14.

Charlton, J.L., Kuzminski, K., Lang, G.M. and Sehon, A.H. "Synthesis, isolation and characterization of conjugates of ovalbumin with monomethoxypolyethylene glycol using cyanuric chloride as the coupling agent.", *Anal. Biochem.*, 165: 114, 1987, incorporated herein by reference in its entirety.)

Monomethoxypolyethylene glycol (2.5 g. mol wt 5590, Union Carbide) was dissolved with warming in anhydrous benzene (40 ml) and a portion of the benzene (20 ml) was removed by distillation to azeotrope off any water in the polymer. Cyanuric chloride {$(CNCl)_3$, 0.83 g, Aldrich, recrystallized from benzene} was added under nitrogen followed by potassium carbonate (0.5 g. anhydrous powdered) and the mixture stirred at room temperature for 15 hours.

The mixture was then filtered under dry nitrogen and the filtrate mixed with anhydrous petroleum ether (ca 50 ml, b.pt. 30-60°C) in order to precipitate the polymer. The polymer was separated by filtration under nitrogen, dissolved in benzene (20 ml) and reprecipitated with petroleum ether. This process was repeated seven times to insure that the polymer was free of any residual cyanuric chloride. The active intermediate was finally dissolved in benzene, the solution frozen and the benzene sublimed away under high vacuum to leave a fine white powder.

Elemental analysis of the intermediate confirmed that it contained 2 chlorine atoms. The intermediate, corresponding to $C_{256.3}H_{307.7}O_{127.2}N_3Cl_2$ with an average molecular weight of 5,738 daltons would have a theoretical composition in percentages of C, 53.65; H, 8.92; N, 0.73; Cl, 1.24; which agrees with its determined composition of C, 53.51; H, 8.89; N, 0.77; Cl, 1.08.

The chloride content of the intermediate was also determined by hydrolysis and titration of the chloride released with silver nitrate. Thus, the activated intermediate (120 mg) was dissolved in water (10 ml) and the pH adjusted to 10 with dilute sodium hydroxide.

After heating at 90°C for two hours, the solution was cooled and the chloride titrated with silver nitrate (0.001N), using a chloride ion selective electrode to indicate the endpoint. The chloride content of the activated intermediate was found to be 2.1, consistent with the structure shown above.

The OVA {40 mg, purified by chromatography on Ultrogel® AcA-54 (LKB, Bromma, Sweden)} was dissolved in sodium tetraborate buffer (4 ml, 0.1 M, pH 9.2) and the activated mPEG added to the solution at 4°C. The amount of activated mPEG was varied to prepare conjugates of differing degrees of polymer substitution. Mole ratios (mPEG/OVA) used to prepare specific conjugates are given in Table 2.

The polymer-protein mixture was stirred for one half hour at 4°C and then one half hour at room temperature. The reaction mixture was desalted by either dialyzing for four days against running distilled water or by passing through a column of Sephadex® G-25 (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

A DEAE-cellulose or DEAE-Sephacryl® (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column (5 cm by 30 cm) was equilibrated with phosphate buffer (0.008 M, pH 7.7). The salt free OVA conjugates were applied in water and the free (unbound) mPEG washed through the column with the pH 7.7 buffer. Free mPEG was detected on thin layer chromatography {Camag (Kieselgel DSF-5, Terochem Lab Ltd, Alberta) eluant 3:1 chloroform/methanol} using iodine vapor for development. After removal of the free mPEG from the ion-exchange column, sodium acetate buffer (0.05 M pH 4.0) was used to elute the conjugate. The conjugate fractions were dialyzed and lyophilized to give the dry conjugates.

Tolerogens of saporin, ricin a chain, birch pollen allergen Bet v 1 and recombinant ragweed allergen R8.1 have also been prepared by the above method, with an induction of tolerance to these antigens.

TABLE 2

Preparation of OA-mPEG$_n$ Conjugates

Conjugates[a]	Preparation ratio[b]	% mPEG[c,e]	%OA[d,e]
OA-mPEG$_{3.2}$	10:1	26	70
OA-mPEG$_{6.6}$	25:1	36	47
OA-mPEG$_{7.6}$	25:1	42	47
OA-mPEG$_{10.6}$	50:1	51	41
OA-mPEG$_{11.9}$	50:1	52.4	38

[a] The degree of substitution, n, is calculated by the formula

$$\frac{\%\ \text{mPEG}}{\%\ \text{OA}} \times \frac{\text{mol wt OA}}{\text{mol wt mPEG}}$$

[b] Mole ratio mPEG:OA based on a molecular weight of 5.740 for mPEG-dichlorocyanurate and 44.460 daltons for OA.

[c] The percentages of mPEG by weight were determined by nuclear magnetic resonance (NMR).

[d] The percentages of protein by weight were determined by the biuret method.

Determination of the immunosuppressive effect of the IgE response of different OVA-mPEG$_n$ conjugates

The results of experiments illustrated in Fig. 8 clearly demonstrate the stringent dependency of the suppressogenicity of mPEG conjugates on their molecular composition. Thus, whereas treatment of groups of four (B6D2)F1 mice each with 50 μg of OVA-mPEG$_{3.2}$, or OVA-mPEG$_{6.6}$, or OVA-mPEG$_{7.6}$ one day prior to intraperitoneal immunization with the sensitizing dose, consisting of 1μg of OVA and 1 mg Al(OH)$_3$, led to essentially complete (99-100%) abrogation of the primary anti-OVA IgE response, as measured --on day 14 after immunization-- by PCA in hooded rats, the more

substituted conjugates, i.e. OVA $mPEG_{10.6}$ and OVA-$mPEG_{11.9}$, inhibited the anti-OVA IgE response, respectively, only to the extent of 94% and 50%. In this and the following examples, the weights of the conjugates given correspond to their protein content.

Long lasting suppression of the IgE response by protein mPEG conjugates in contrast to a transient suppressive effect of unconjugated protein

Even unmodified OVA was capable of downregulating the primary IgE response in relation to the response of control mice which had received PBS instead of OVA or conjugates. In this experiment three groups of four (B6D2)F1 mice each received phosphate buffered saline, or 50 μg of OVA-$mPEG_{4.5}$ or 50 μg of OVA. All animals were bled on day 10, 14, 21, 27, 35, 42 and 49 and their IgE titers were determined by PCA in hooded rats. As illustrated in Table 3, it is important to point out that whereas the suppressogenic effect of OVA-mPEG conjugates was long-lasting, the down regulating effect of free OVA was of short duration and, in actual fact, its administration predisposed the animals to an anamnestic response which reached, after booster immunization (administered on day 28), IgE antibody levels equivalent to those of control animals which had received PBS and the two sensitizing doses of one antigen. The results given in Table 3 clearly demonstrate that a tolerogenic conjugate injected prior to repeated administration of the corresponding free protein essentially abrogated the immune response.

TABLE 3

Effect of administering 50 μg of OA-$mPEG_{4.5}$ or of free OA one day prior to immunization

Day of bleeding after primary immunization	PCA titers for groups of mice treated with		
	PBS	OA	OA-$mPEG_{4.5}$
10	5,120	40	< 4
14	1,940	40	< 4
21	1,280	40	< 4
27	640	40	< 4
35	1,920	1,920	160
42	2,560	1,280	160
49	5,120	N.D.*	160

On day 28 all three groups received a booster dose of the sensitizing OA preparation.
* N.D. = not determined.

The effect of different doses of the tolerogen on the IgE response

Each OVA-mPEG conjugate was injected into groups of 4 mice each at the four doses of 10 μg, 50 μg, 150 μg and 600 μg. The control group of mice received PBS as placebo.

As is evident from Fig. 9, treatment with different conjugates at doses of 10 μg and 50 μg per mouse revealed marked differences in their suppressogenic capacity. It is also to be noted that at a dose of 150 μg, all conjugates were highly suppressive and at 600 μg, all the compounds tested suppressed completely the IgE response.

The effect of different doses of the tolerogen on the IaE response

The sera used in Fig. 10 to illustrate the effect of tolerogenic conjugates on the IgG response were the same as those used in Fig. 8. As illustrated in Fig. 10, administration of 50 μg of OVA-$mPEG_{7.6}$ resulted in the maximal suppression, i.e. of the order of 98% of the primary anti-OVA IgG response, which was determined 14 days after the first injection of the sensitizing dose of OVA by a radio-immunoassay employing the paper radio immunosorbent procedure (Kelly, K.A. et al.; J. Immunol. Meth. 39 (1980) p. 317-33) utilizing OVA bound to the paper and with $_{125}$I-labelled affinity purified sheep antiserum to mouse IgG.

Example 4

The suppressive effect of OVA-$mPEG_{10}$ on IgM, IgG, and IgE plaque forming cells (PFC) in spleen and lymph nodes. One mg of OVA-$mPEG_{10}$ (containing 10 mPEG groups with an average mol wt of 10,000 daltons, which were coupled per OVA molecule by the succinic anhydride method (Wie, S.I. et al.,

Int. Archs. Allergy appl. Immun. 64, 84 1981)) or PBS was administered intraperitoneally to each group of four (B6D2)F1 mice each one day prior to immunization with 1 μg of DNP_3-OVA in 1 mg $Al(OH)_3$.

On several days thereafter the spleen, as well as the mesenteric, parathymic and inguinal lymph nodes were removed and assayed for IgM, IgG, and IgE anti-DNP PFC (Rector, E.S. et al., Eur. J. Immunol. 10, p. 944-49 (1980). In Table 4 are given the numbers of PFC in the above tissues 10 days after immunization; from these data it is evident that treatment with this tolerogen markedly reduced the number of IgM, IgE, and IgG PFC in all tissues examined. Therefore, these results support the claim that the tolerogens shut off the immune response rather than neutralize the circulating antibodies.

TABLE 4

The effect of OA-$mPEG_{10}$ on the suppression of IgM, IgG, and IgE plaque forming cells (PFC) in spleen and lymph nodes

Antibody Class	Treatment	Anti-DNP PFC per 10^8 cells from different tissues* Spleen	Parathymic Nodes	Mesenteric Nodes	Inguinal Nodes
IgM	PBS	2,150	2,950	Nd	Nd **
	OA-mPEG	900	200	Nd	Nd
IgG	PBS	15,350	78,550	5,000	Nd
	OA-mPEG	Nd	1,300	Nd	Nd
IgE	PBS	10,410	16,530	11,140	300
	OA-mPEG	500	950	400	Nd

* Each tissue sampling represents a pool from 4 mice

The above experiments establish the immunosuppressive effects discussed above and the effects at various dosages.

In addition, utilizing the hu-PBL-SCID mice, it was demonstrated that in accordance with the phenomenon of

"linked immunological suppression", cross-specific suppression of the human antibody response could be induced to murine mAbs which differ in their antigen binding specificities from those of the murine mAbs which had been incorporated into the tolerogenic conjugates, on condition that both mAbs shared the same heavy and light chains. Thus, that pan-specific suppression of the "human" antibody responses against murine monoclonal antibodies (i.e., HAMA responses) of the IgG class could be achieved with 8 tolerogenic mPEG preparations, each consisting of one of the 4 gamma chains and of one of the two types of light chains of murine IgG (Bitoh, S., Lang, G.M., Kierek-Jaszczuk, D., Fujimoto, S. and Sehon, A.H. Specific immunosuppression of human anti-murine antibody (MAMA) responses in hu-PBL-SCID mice. Hum. Antibod. Hybridomas 4:144-151, 1993).

Thus, in accordance with the present invention, prior to beginning of gene therapy, i.e., prior to insertion of a new gene into a host which is required for expression of a protein beneficial to the host, e.g., one of the deficient clotting factors or enzymes, it is essential to render the host tolerant to the protein in question by the use of the invention described.

Example 5

The expressed protein material of the cystic fibrosis transmembrane conductance regulatory gene (CFTR) (Genzyme, Cambridge, Massachusetts) for the treatment of cystic fibrosis is dissolved in sodium tetraborate buffer (4 ml, 0.1 M, pH 9.2) and the activated mPEG added to the olution at 4°C.

The amount of activated mPEG is varied to prepare conjugates of differing degrees of polymer substitution. Different mole ratios (mPEG/gene product) are used to prepare specific tolerogenic conjugates as described earlier.

The polymer-gene product mixture is stirred for one half hour at 4°C and then one half hour at room temperature. The reaction mixture is desalted by either dialyzing for four days against running distilled water or by passing through a column of Sephadex® G-25 (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

A DEAE-cellulose or DEAE-Sephacryl® (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column (5 cm by 30 cm) is equilibrated with phosphate buffer (0.008 M, pH 7.7). The salt free mPEG conjugates of the cystic fibrosis gene product are applied in water and the free (unbound) mPEG washed through the column with the pH 7.7 buffer.

Free mPEG is detected on thin layer chromatography {Camag Kieselgel DSF-5, Terochem Lab Ltd, Alberta) eluant 3:1 chloroform/methanol} using iodine vapor for development.

After removal of the free mPEG from the ion-exchange column, sodium acetate buffer (0.05 M, pH 4.0) is used to elute the conjugate. The conjugate fractions are dialyzed and lyophilized to give the dry conjugates. Conjugates of the CFTR gene are administered to a patient at least one day prior to transfer of the cystic fibrosis transmembrane conductance regulator gene to lung tissue using recombinant adenoviral vectors or liposomes.

Example 6

The expressed protein material of the low density lipoprotein receptor (LDLr) gene used in the treatment of familial hypercholesterolemia is dissolved in sodium tetraborate buffer (4 ml, 0.1 M, pH 9.2) and the activated mPEG added to the solution at 4°C. The amount of activated mPEG is varied to prepare conjugates of differing degrees of polymer substitution.

Different mole ratios (mPEG/gene product) is used to prepare specific tolerogenic conjugates as described earlier. The polymer-gene product mixture is stirred for

one half hour at 4°C and then one half hour at room temperature. The reaction mixture is desalted by either dialyzing for four days against running distilled water or by passing through a column of Sephadex® G-25 (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

A DEAE-cellulose or DEAE-Sephacryl® (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column (5 cm by 30 cm) is equilibrated with phosphate buffer (0.008 M, pH 7.7).

The salt free mPEG conjugates of the LDLr-gene products are applied in water and the free (unbound) mPEG washed through the column with the pH 7.7 buffer. Free mPEG is detected on thin layer chromatography {Camag (Kieselgel DSF-5, Terochem Lab Ltd, Alberta) eluant 3:1 chloroform/methanol} using iodine vapor for development.

After removal of the free mPEG from the ion-exchange column, sodium acetate buffer (0.05 M, pH 4.0) is used to elute the conjugate. The conjugate fractions are dialyzed and lyophilized to give the dry conjugates.

Conjugates of the LDLr gene product are administered to a patient. Hepatocytes are grown in the laboratory and genetically altered with a murine retroviral vector containing LDLr gene. The cells are reinfused through the hepatic artery to the liver of the patient at least one day after administration of the conjugate.

Table 5 shows a list of gene therapy systems which have been approved by the Recombinant DNA Activities Committee of the National Institutes of Health. However, no consideration appears to have been given to overcoming the potential complications due to the host mounting an immune response against the respective gene products.

Thus the present invention can readily be adapted to any gene therapy protocol and is generally applicable to the administration of any therapeutic immunogenic material and not just the specific examples listed above.

Gene therapy according to the existing art may be

applied to somatic cells or germ line cells by methods known such as gold electroporation, microinjection or jet injection, or other methods as set forth in Sambrook et al. "Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989)" incorporated herein by reference in its entirety. Thus the invention provides for a method for treating by gene therapy a mammal with a therapeutic amount of a biologically active antigenic material or its expression product. To retain the effectiveness of said antigenic material(s) from counteraction by an antibody(ies) produced against it(them); it is essential to suppress the capacity of the recipient of the gene to mount an antibody response(s) to said biologically active antigenic material(s).

This method comprises conducting gene therapy by administering to a mammal an immunosuppressive effective amount of a tolerogenic conjugate consisting of a protein coupled to monomethoxypolyethylene glycol (mPEG) having a molecular weight of about 2,000-10,000 daltons, wherein administration of said tolerogenic conjugate is at least one day prior to administration of a gene therapy vector encoding a gene for a protein, wherein said protein is identical to said protein which is coupled to mPEG, and wherein said method results in the suppression of an immune response and in the development of tolerance to the protein expressed by said gene encoded by said gene therapy vector.

Alternatively the method of conducting gene therapy includes a) administering to a mammal an immunosuppressive effective amount of a tolerogenic conjugate consisting of a protein conjugated to monomethoxypolyethylene glycol (mPEG) having a molecular weight of about 2,000 to 10,000 daltons, wherein administration of said tolerogenic conjugate is at least one day prior to administration of DNA, RNA or mRNA and encoding a protein administered for gene therapy, wherein the encoded protein is identical to said protein

which is conjugated to mPEG, and wherein said method results in the suppression of an immune response and in the development of tolerance to encoded protein of said DNA, RNA or mRNA administered for gene therapy. The method suppresses the formation of about 98% of antibodies against said antigenic genetic material or its product.

Example 7

The method of suppression of the humoral and cellular immune responses of the invention finds application in specific area of gene and protein replacement therapy. In particular, patients with Hemophilia A are from birth deficient of the gene which serves as the template for the production of clotting Factor VIII (F.VIII). The current replacement therapy involves the frequent administration of F.VIII, which is isolated from blood of normal volunteers or synthesized by recombinant technology. Either of these therapies is extremely expensive and, most importantly, the delay between the onset of bleeding and administration of the factor may lead to tissue damage.

J. Walter and K.A. High report encouraging results obtained for the *in vivo* and *ex vivo* synthesis of F.VIII (and of F.IX) by the latest methods of gene therapy with the aid of naturally occurring animal (mouse and dog) models of the disease. In vivo gene therapy is the treatment of choice, since it would provide sustained synthesis of the missing clotting factor and obviates the need for frequent treatments. Therapies involving the administration of natural or recombinant clotting factor, the transgenic clotting factor, as well as the proteins expressed by the companion adenoviral vector, elicit the formation of the respective anti-factor antibodies ("inhibitors") and anti-vector antibodies which may undermine the effectiveness of gene therapy.

The transgenic protein as well as the vector

protein(s), in addition to being excreted as such by the transfected cells, may also be degraded with the cell into peptides incorporating some of the epitopes of the protein(s). These foreign peptides, when presented in association with MHC molecules on the cell membrane, are believed to elicit the production of cytotoxic cells capable of destroying the transfected cells.

Immune response can be blunted by a variety of immunosuppressive regimens, which have been reviewed in relation to treatment of hemophilia by Bertrop, et al in an article based on the discussions at a joint WHO and World Federation of Hemophilia meeting (2). Most of these therapies, as in the case in order transplantation, are nonspecific and involve the continual administration of immunosuppressive drugs which are expensive and may have deleterious side effects.

The same gene therapy protein complicating features apply also to adenoviral vector proteins. Although it is possible that less immunogenic or totally non-immunogenic adeno-associated virus (AAV) vectors will be developed and that vectorless insertion of transgenes into the host's DNA are achieved, the immunogenicity of the transgenic protein still represents a serious problem. Indeed, this phenomenon is evidence in canine anti-Factor VIII antibody generated in hemophilic animals treated with a homologous factor VIII transgene.

Evidence for the utility of his discovery for the induction of long term antigen (Ag)-specific suppression of both antibody and cytotoxic cell responses by pretreatment of the host with the tolerogenic conjugate of the particular Ag and an optimal number (n) of molecules of monomethoxy-polyethylene glycol (mPEG), i.e., $Ag(mPEG)_n$ is presented below. The administration of only a single dose of 100-200 μg of tolerogenic mPEG conjugates of a variety of antigens sufficed to induce specific long term suppression

of the immune response in mice and rats, in spite of multiple injections of the respective antigens over extended periods. Most importantly, mPEG has been shown to be a biocompatible polymer and has been used for the synthesis of diverse pharmaceutical products.

The method described below is readily adapted for induction of (F.VIII)-specific suppression of the immune response in (F.VIII)-deficient recipients irrespective of the origin of F.VIII.

Prevention of Therapeutic Complications due to the Immunogenicity of Recombinant Biologic Response Modifiers (BRMs)

A major challenge for the biotechnology of potentially therapeutic recombinant BRMs is to overcome their immunogenicity, as is the case for natural foreign proteins (e.g., monoclonal antibodies, enzymes, toxins, hormones, heterologous F.VIII). The therapeutic effectiveness of even the corresponding chimeric or humanized recombinant proteins is undermined by their immunogenicity which is often due to only minor conformational differences with respect to the three-dimensional structures of their corresponding natural progenitors.

Consequently, administration of some recombinant BRMs elects the production of complementary, neutralizing and/or blocking antibodies by the hose. These antibodies intercept the BRMs and prevent them from reaching their target cells. Moreover, depending on the class of antibodies elicited, the patient may develop serum sickness, renal and hepatic toxicity, and even anaphylactic shock in severe cases.

The inventors have developed a method for conversion of a variety of immunogenic proteins or immunogenic fragments of the proteins (P) to tolerogenic derivatives. This conversion involves the coupling of an optimal number (n) of molecules of monomethoxypolyethylene glycol (mPEG) onto the

protein antigen in question. The protocol for the effective induction of specific immunosuppression in mice and rats consists of two steps:

Step I: Injection of tolerogenic conjugates of the appropriate immunogenic BRM, i.e. $P(mPEG)_n$;

Step II: Administration of the unmodified P, about 7 days after injection of the immunosuppressive $P(mPEG)_n$. Thereafter, unmodified biologically active P can be injected repeatedly over extended periods without further injection of $P(mPEG)_n$.

For example, in mice suppression of antibody responses to heat aggregated human monoclonal (myeloma) IgG, referred to as HaHIgG, was shown to persist up to 540 days in spite of multiple injections of HaHIgG at different intervals over this span of time.

The reason for the interval of about 7 days betweens Steps I and II is to allow propagation of P-specific suppressor T (Ts) cells which are activated by $P(mPEG)_n$ (4). Additional injections of the unmodified P maintain the proliferation of these cells, which suppress the specific T helper cells that also recognize the epitopes of the same P, though not necessarily the same epitopes as those recognized by the Ts cells.

It is also noted that pretolerization of mice to a given protein Ag_A by treatment with $Ag_A(mPEG)_n$ results in their becoming immunologically unresponsive to an unrelated Ag_B, on condition that Ag_B is injected into these mice in the form of a covalent adduct with Ag_A, i.e., as Ag_A-Ag_B, but not as a mixture with Ag_A. This cognate phenomenon of *"linked immunological suppression"* is also conferred on naive mice by treating them first with TsF_A nd then with the Ag_A-Ag_B adduct. Obviously, any clone of Ts cells recognize only one epitope (i.e. one antigenic determinant) of the respective high molecular weight multi-determinant protein.

Application to suppression of the antibody response of the human lymphoid system and gene therapy

Experiments were performed in hu-PBL-SCID mice. The abbreviation "hu-PBL-SCID mouse" denotes a mouse with severe combined immunodeficiencies, which has been engrafted with human peripheral blood leucocytes. This system represents the closest *in vivo* model for a functional human lymphoid system. The inventors demonstrated that (i) mPEG conjugates of a foreign P, i.e., murine mAb, induced specific suppression of human anti-P antibodies, (ii) this suppression was due to the generation of human P-specific $CD8^+$ T cells, and (iii) the suppression was transferable with these human Ts cells into secondary naive recipient SCID mice that had been engrafted with the leucocytes of the original donor of the cells (9, 10).

In addition, utilizing the hu-PBL-SCID mice, it was demonstrated that, in accordance with the phenomenon of "linked immunological suppression', cross-specific suppression of the human antibody response could be induced to murine mAbs which differ in their antigen binding specificities from those of the murine mAbs which had been incorporated into the tolerogenic conjugated, on condition that both mAbs shared the same heavy and light chains.

Thus, it was concluded that pan-specific suppression of the "human" antibody responses against murine monoclonal antibodies (i.e., HAMA responses, including anti-diotypic responses) of the IgG class could be achieved with only eight tolerogenic mPEG preparations, each consisting of one of the four gamma chains and one of the two types of light chains of murine IgG.

The two-step method described above is demonstrated to be suitable for suppressing the activation of cytotoxic T cells (CTLs), as is the case for gene therapy involving the transfection of a "new" gene into an immunocomponent patient who has been deficient from birth of the particular gene

required to express the corresponding protein. It is established that the same mPEG conjugate, i.e., OVA(mPEG)$_{10}$, had the dual capacity of suppressing the antibody response and the activation of Ag-specific CTLs (5). For these experiments, the gene therapy model system consisted of E.G7-OVA target cells, which had been generated by Dr. M. Bevan by transfecting syngeneic (C57BL/6) Ia-EL-(H-2b) thymoma cells with the OVA cDNA gene. Thus, whereas control mice which had been primed with OVA in CFA developed OVA-specific CTLs that lysed the target cells, induction of both Abs and CTLs to OVA was abrogated in mice which had been immuno-suppressed by pretreatment with tolerogenic OVA(mPEG)$_{10}$ conjugated, but not with unmodified OVA.

Experiments with Human Factor VIII (hF.VIII)

Preparation of hF.VIII-mPEG conjugates

Recombinant hF.VIII has a molecular weight of 330,000 and consists of 2,332 amino acids, 160 of which are lysines (13). The first conjugate will be synthesized using one of our proven methods for the preparation of tolerogenic derivatives of diverse protein antigens with molecular weights in the range of 51,000-150,000 Da, vis., recombinant human insulin (rhI), saporin, chain A of ricin, a major birch pollen allergen (Bet v 1), a recombinant ragweed allergen (r8.1), ovalbumin (OVA), and human and mouse IgG. With the exception of rhI, the common feature shared by the other tolerogens is that their compositions consisted of 2 to 3 mPEG molecules (coupled via ϵ-amino groups of lysines) per 100 amino acids. Human F.VIII has 6-7 lysines per 100 amino acids and it is anticipated, on the basis of our experience with other tolerogens, that at least 50% of them would be available for coupling.

Each of the aggregate-free proteins is reacted with a large excess of the activated mPEG derivative, viz., mPEG p-nitrophenyl carbonate (mPEG-NPC), which reacts preferen-

tially with ε-amino groups and which was custom-synthesized by Shearwater Polymers Inc. (Huntsville, AL) utilizing a highly purified preparation of mPEG (average Mr-3200 Da) containing less than 2% of diols. The mPEG-NPC is used in a large molar excess in relation to the total lysine content of each protein because of its substantial hydrolysis at the relatively high pH which is required for coupling with ϵ-NH_2 groups.

mPEG-NPC is used in an 8-fold higher molar concentration than that of the lysines of a given protein. For example, 47 mg of OVA corresponding to 1mM of OVA (which contains 19 lysines per molecule of OVA) is reacted with 486.4 mg (152 mM) of mPEG-NPC for the synthesis of tolerogenic OVA$(mPEG)_n$ conjugates containing an average of 10-11 molecules of mPEG per molecule of OVA; the subscript n, refers to the average degree of conjugation. For the synthesis of tolerogenic mPEG conjugates of mouse or human IgG, 150 mg of IgG corresponding to 1 mM of IgG and containing on the average 90 lysines per molecule of IgG, was reacted with 2.3 grams of mPEG-NPC. The resulting tolerogenic IgG$(mPEG)_n$ conjugates contained on the average 25 to 35 mPEG molecules per molecule of IgG.

For the synthesis of hF.VIII$(mPEG)_n$ conjugated, hF.VIII is dissolved at 10 mg/ml in 0.1 M borate buffer, pH 9.7 and then mixed quickly with mPEG-NPC which is dissolved in an identical volume of double distilled water (DDW). Prior to initiating this procedure it is established that hF.VIII is stable at pH 9.7 in borate buffer. The reaction mixture is transferred to a dialysis bag, which should then be suspended in 4 liters of 0.05M borate buffer (pH 9.7) and the reaction is continued with constant stirring for 1 hour at room temperature and overnight in the cold; the large volume of buffer outside the dialysis bag serves as a pH-stat. Finally, for the isolation of the conjugate, the content of the dialysis bag is applied onto a Pharmacia

BioPilot gel filtration column which has been equilibrated with DDW. The conjugate is in the void volume, followed by (i) hydrolyzed mPEG-NPC, and (ii) the buffer salts and the p-nitrophenyl released in the reaction. The isolated conjugate is lyophilized and stored at -20°C.

The method of synthesis of the tolerogenic hF.VIII$(mPEG)_n$ conjugates in a pure form involves a series of steps, including isolation of the pure conjugate by gel filtration chromatography, which leads to yields of the order of 50-70%.

Immunization of normal inbred BALB/C mice, normal out-bred CD-1 mice and normal outbred Sprague-Dawley rats with hF.VIII in the presence of adjuvant

In view of the low immunogenicity of hF.VIII, to ensure a consistent immune response in mice and rats, hF.VIII is administered in an adjuvant. Five to seven week old female mice of both strains receive two ip injections of a suspension of hF.VIII in $Al(OH)_3$ at an interval of 21 days. The strain of mice which mount the most consistent anti-hF.VIII antibody titer is selected for further experiments with hF.VIII in $Al(OH)_3$. Freund's adjuvant is used with the Sprague-Dawley rats because this adjuvant has been proven to induce high titered Ab responses to hF.VIII in this strain of rats. The rats are immunized sc with hF.VIII in Freund's adjuvant; the first injection contains complete Freund's adjuvant and the second injection after an interval of 3 weeks contains incomplete Freund's adjuvant. The mice and rats are bled at weekly intervals and the antibody production is determined by ELISA.

Repeated treatment of normal mice and rats with hF.VIII in the absence of adjuvant

This immunization regimen resembles more closely that administered to hemophilic patients. Both BALB/C and CD-1 ice and the Sprague-Dawley rats are injected with hF.VIII

without adjuvant and their sera is assayed for the presence of antibody by ELISA. The dose of hF.VIII and the interval of time between repeated injections of hF.VIII is refined in this series of experiments. The presence of antibody with a significant titer in at least 50% of the animals justifies continuation with this immunization strategy.

Induction of tolerance

Several hF.VIII(mPEG)$_n$ conjugates differing in "n" are be synthesized. Each of the hF.VIII(mPEG)$_n$ conjugates is tested for its degree of tolerogenicity in mice as described In step I, 4 groups of 4 mice each receive ip, respectively, 100, 200, 400 and 800 mg of hF.VIII(mPEG)$_n$. The mice are be given the first immunizing dose of hF.VIII in adjuvant 7 days later, and are re-immunized after 21 days. control mice (4 mice per group) are injected ip with unmodified hF.VIII, or with diluent in lieu of conjugate. All mice are bled at weekly intervals after primary immunization for assaying their sera by ELISA.

Next, the effective tolerogenic dose of each conjugate required for suppressing the immune response when hF.VIII is injected with or without adjuvant is established by the above protocol. Control animals are given unmodified hF.VIII or diluent in lieu of conjugate. The animals are be bled at weekly intervals and the sera assayed by ELISA. The most immunosuppressive conjugate in mice are tested in Sprague-Dawley rats. In accordance with the procedures used for mice, the conjugate is injected 7 days prior to primary immunization. Four groups of 6 rats each receive ip, respectively, 100, 200, 400 and 800 mg of hF.VIII(mPEG)$_n$. Two control groups of 6 rats each are injected ip, respectively, with unmodified hF.VIII or diluent in lieu of conjugate. The rats are re-immunized 21 days after the primary immunization. All rats are bled at weekly intervals after primary immunization for the determination of their anti-hF.VIII levels by ELISA.

Immunosuppression of an ongoing immune response to hF.VIII by a protocol involving a combination of hF.VIII(mPEG)$_n$ conjugate and mycophenolic acid or other immunosuppressive drug.

It was established in experiments, utilizing OVA as the antigen and mycophenolic acid (MPA), as the immunosuppressive drug, that (i) whereas the ongoing antibody response was abrogated while MPA was administered, the response reappeared after the administration of MPA had been discontinued, and (ii) administration of MPA did not affect the induction of suppression by mPEG conjugates.

Therefore, the dose of MPA required for abrogation of an ongoing immune response to hF.VIII in mice and in Sprague-Dawley rats is established.

Considering that some of the patients may have been given hF.VIII prior to receiving the tolerogenic mPEG-conjugate of hF.VIII it is important to establish if co-administration of an immunosuppressive dose of MPA or other immunosuppressive drug and of a tolerogenic dose of hF.VIII(mPEG)$_n$ may lead to long-term suppression of the ongoing immune response to hF.VIII.

Tolerance in hemophilic mice

Recent studies in hemophilic mice indicate that they too develop neutralizing antibodies to human factor VIII upon exposure to this protein (17). With this as background, as the tolerogenic capacity of one or more hF.VIII(mPEG)$_n$ conjugates is established their immunosuppressive capacity in hemophilic mice when administered prior to injection of hF.VIII is tested. The degree of immunosuppression both by ELISA and in terms of the effect on clotting time as measured by the Bethesda test is established. If co-administration of MPA or other immunosuppressive drug with the tolerogenic hF.VIII(mPEG)$_n$ conjugate leads to effective suppression of an ongoing anti-

hF.VIII antibody response in mice and rats, the usefulness of this regimen in hemophilic mice producing "inhibitors" is established. Hemophilic dogs are also used in the investigations.

Example 8

Transplantation Example

Rejection of transplanted cells and organs is also an application for the mPEG strategy. Rejection results from the recognition of proteins encoded by the MHC, HLA in humans, by $CD4^+$ and/or $CD8^+$ T cells. Many antigens have been expressed in large amounts by recombinant technology. Purified HLA antigens can be modified with mPEG and the corresponding conjugates injected into patients prior to organ or cell transplantation. For antigens that have not yet been identified or for which recombinant proteins are not yet available, the inventors can modify peripheral blood cells or membranes from these cells with mPEG. This is feasible since it has been shown that erythrocytes can be modified with mPEG. MHC specific tolerance is induced with mPEG modified antigens; hence suppression of the immune response and of the rejection of transplants by prior pretreatment by the appropriate MHC-mPEG conjugate is possible with this treatment or in conjunction with reduced doses of immunosuppressive drugs that have various side effect, including inhibition of all immune responses.

The invention provides a method of preventing an immune rejection of organ transplants comprising administering an effective amount of Ag(mPEG) conjugate to induce tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses.

The present invention targets mPEG to antigen-specific T cells, including $CD8^+$ T cells. The inventors have used published methods for stimulating $CD8^+$ T cells with exogenous antigens by delivery in adjuvant to test this

idea. Hence, mPEG-OVA complexes were injected before priming mice with OVA in Freund's complete adjuvant. About two weeks later, mice were sacrificed and their spleen cells assayed for development of OVA specific cytolytic T cells (CTL).

The results showed that mPEG inhibited priming for OVA-specific CTL and thus caused tolerance that prevented normal stimulation. In addition, this technique is extended to coupling of various cells with mPEG. Cells conjugated with mPEG are used to induce tolerance to specific MHC antigens of a given organ graft donor prior to organ transplantation.

Example 9

Autoimmunity Example

The inventors provide approaches that are effective to induce tolerance and thus could be used as alternatives to treat autoimmunity.

mPEG modification of such antigens render them tolerogenic and have the potential to reverse these autoimmune diseases. This therapy can be extended to any autoimmune disease, in which a tissue specific antigen can be identified. It is believed that generations of a particular number of Ts cells may reverse these autoimmune diseases. This therapy may be extended to any autoimmune disease assuming that the culprit tissue specific antigen is identified, isolated or synthesized for preparation of the corresponding mPEG conjugate.

Several autoimmune diseases are known to involve cytokine producing $CD4^+$ T cells and/or $CD8^+$ T cells that recognize auto-antigens. Such T cells can transfer disease in normal syngeneic recipients, as is the case within the same strain of mice or rats. In experimental multiple sclerosis, and the related model of experimental autoimmune encephalomyelitis (58), T cells have been shown to recognize

several antigens. Such T cells can transfer different disease into normal syngeneic recipients.

In multiple sclerosis and in experimental autoimmune encephalomyelitis, T cells have been shown to recognize several antigens that are restricted in expression to the central nervous system. These include: myelin basic protein, proteolipid protein and myelin oligodendrocyte antigen. T cells recognizing insulin, glutamic acid decarboxylase, and other, as yet unidentified antigens expressed by beta cells, have been isolated from non-obese diabetic (NOD) mice, BB rats, and diabetic humans.

mPEG treated cells can be used to induce tolerance to certain cells in the body that are known to be the targets of autoimmunity. For example, beta cells from the pancreas are modified with mPEG and injected into nonobese diabetic (NOD) mice before or after the appearance of spontaneous diabetes.

The tolerogenic properties of the conjugates of the invention have the capacity to inactivate (in vivo in PCA reactions) IgE-sensitized rat skin mast cells, and B_E cells. (24). Injection of $OVA(mPEG)_{10}$ into skin sites inhibited the release of mediators of anaphylaxis on subsequent injection of 1 mg DNP_{44}-BSA and 0/1mg. of Bet v 1. In contrast, injection with unmodified OVA or unrelated mPEG conjugate prior to i.v. challenge with DNP_{44}BSA or Bet v 1, did not affect the PCA reactions to either of these two antigens.

The conjugates of the invention, where the antigen is an allergen, inactivate mast cells. This resulted in specific suppression of a primary immune response after administration of the conjugates of the invention. The allergenicity of the conjugates of the invention are 10-500 fold lower than that of the original allergen.

The method of the invention may be practiced to treat a condition selected from allergies and autoimmune diseases by

inducing tolerance to an antigen (Ag) or self-protein or autoantigen, in both humoral and cell mediated immune responses comprising administering an effective amount of Ag(mPEG) conjugate to induce tolerance to an antigen (Ag).

The invention treats organ-specific autoimmune diseases in animal and rejection of DNA transfected cells of their products by administration of mPEG conjugates of autoantigens selected from the group consisting of collagen-induced arthritis by type II collagen, experimental autoimmune encephalomyelitis by myelin basic protein, and diabetes in NOD mice by insulin to induce tolerance to an antigen (Ag) in both humoral and cell mediated immune responses.

The principles of the invention provide a method of treating organ-specific autoimmune diseases in animal and rejection of DNA transfected cells of their products comprising administration of mPEG conjugates of autoantigens selected from the group consisting of collagen-induced arthritis by type II collagen, experimental autoimmune encephalomyelitis by myelin basic protein, and diabetes in NOD mice by insulin to induce tolerance to an antigen (Ag) in both humoral and cell mediated immune responses.

Example 10

A method of treating established allergic responses or established autoimmune disease states involves a combination therapy as follows:

i) wipe out the established immune response of the host to all antigens by pharmacological immunosuppressive agents for a period of about 2 to about 3 weeks, without destruction of the stem cells, and

ii) treatment of the "immunologically revirginized" patient with mPEG conjugates of the auto-antigens in anticipation of generating specific Ts cells which would suppress the induction of the Th cells involved in the

auto-immune response.

Any known immunosuppressive drugs may be used for step i) such as those which specifically target suppression of T cells or B cells or both. Alternatively, step i) may be substituted by a bone marrow or stem cell transplant.

Thus the invention provides promising results in experimental animal models that conjugates of immunogenic Ag and mPEG provide for the development of novel therapeutic and clinical applications. mPEG derivatives of naturally derived protein Ag also suppress the autoimmunity and prevent the onset of autoimmune diseases. The administration of mPEG conjugates of immunogenic proteins, such as recombinant lymphokines (53) or xenogeneic enzymes (54,55), has proven to be a safe procedure in man. Hence, the clinical applications of mPEG conjugates of diverse therapeutic agents is increased.

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TABLE 5

Disorder (gene used)	*Cells altered (vector)*
Adenosine deaminase deficiency[a] (ADA)	T-cells and stem cells (retroviral)
Brain tumors (MDR-1)	Stem cells (retroviral)
Brain tumors (primary and metastatic) (HS-*tk*)	Tumor cells (retroviral)
Brain tumors (primary)[a] (HS-*tk*)	Tumor cells (retroviral)
Brain tumors (primary and metastatic) (HS-*tk*)	Tumor cells (retroviral)
Brain tumors (primary and metastatic) (HS-*tk*)	Tumor cells (retroviral)
Brain tumors (primary) (anti-sense IGF-1)	Tumor cells (DNA transfection)
Brain tumors (primary)[a] (HS-*tk*)	Tumor cells (retroviral)
Brain tumors (primary and metastatic) (HS-*tk*)	Tumor cells (retroviral)
Breast cancer (IL-4)	Fibroblasts (retroviral)
Breast cancer (MDR-1)	Stem cells (retroviral)
Breast cancer (MDR-1)	Stem cells (retroviral)
Colorectal cancer (IL-4)	Fibroblasts (retroviral)
Colorectal cancer (IL-2 or TNF-α gene)	Tumor cells (retroviral)
Colorectal cancer (HLA-B7 and β2-microglobulin)	Tumors cells (liposomes)
Colorectal cancer (IL-2)	Fibroblasts (retroviral)
Cystic fibrosis[a] (CF TR)	Respiratory epithelium (adenoviral)
Cystic fibrosis[a] (CF TR)	Respiratory epithelium (adenoviral)
Cystic fibrosis (CF TR)	Respiratory epithelium (liposomes)
Cystic fibrosis[a] (CF TR)	Respiratory epithelium (adenoviral)
Cystic fibrosis[a] (CF TR)	Respiratory epithelium (adenoviral)
Cystic fibrosis[a] (CF TR)	Respiratory epithelium (adenoviral)
Familial hypercholesterolemia[a] (LDLr)	Liver cells (retroviral)
Gaucher disease[a] (glucocerebrosidase)	Stem cells (retroviral)
Gaucher disease[a] (glucocerebrosidase)	Stem cells (retroviral)
Gaucher disease[a] (glucocerebrosidase)	Stem cells (retroviral)
Gaucher disease (glucocerebrosidase)	Stem cells (retroviral)
HIV infection (Mutant Rev)	T cells (retroviral)
HIV infection (HIV-1 III *env*)	Muscle (retroviral)
HIV infection (HIV-1 IIIB Env and Rev)	Muscle (retroviral)
HIV infection (HIV-1 ribozyme)	T cells (retroviral)
Leptomeningeal carcinomatosis (HS-tk)	Tumor cells (retroviral)
Malignant melanoma (IL-4)	Tumor cells (retroviral)
Malignant melanoma (IL-2)	Tumor cells (retroviral)
Malignant melanoma (IL-2)	Tumor cells (retroviral)
Malignant melanoma (IL-2)	Tumor cells (retroviral)
Malignant melanoma (IL-4)	Fibroblasts (retroviral)
Malignant melanoma (HLA-B7)	Tumor cells (liposomes)

Disorder (gene used)	*Cells altered (vector)*
Malignant melanoma (HLA-B7 and β_2-microglobulin)	Tumor cells (liposomes)
Malignant melanoma (TNF-α or IL-2)	T cells or tumor cells (retroviral)
Malignant melanoma (interferon-γ)	Tumor cells (retroviral)
Malignant melanoma (B7)	Tumor cells (retroviral)
Neuroblastoma[a] (IL-2)	Tumor cells (retroviral)
Non-small cell lung cancer (p53 or antisense K-ras)	Tumor cells (retroviral)
Ovarian cancer (HS-*tk*)	Stem cells (retroviral)
Ovarian cancer (MDR-1)	Stem cells (retroviral)
Renal cell carcinoma (IL-2)	Tumor cells (retroviral)
Renal cell carcinoma (IL-4)	Fibroblasts (retroviral)
Renal cell carcinoma (TNF-α or IL-2)	Fibroblasts (retroviral)
Renal cell carcinoma (GM-CSF)	Tumor cells (retroviral)
Small cell lung cancer (IL-2)	Tumor cells (DNA transfection)
Solid tumors (HLA-B7 and β2-microglobulin)	Tumor cells (liposomes)

Disorder	Location
Brody myopathy (1)	Chr.16
Burkitt lymphoma (3)	8q24.12-q24.13
C1q deficiency (1)	1p36.3-p34.1
C1r/C1s deficiency, combined (1)	12p13
C2 deficiency (3)	6p21.3
C3 deficiency (1)	19p13.3-p13.2
C3b inactivator deficiency (1)	4q25
C4 deficiency (3)	6p21.3
C5 deficiency (1)	9q34.1
C6 deficiency (1)	5p13
C7 deficiency (1)	5p13
C8 deficiency, type I (2)	1p32
C8 deficiency, type II (2)	1p32
C9 deficiency (1)	5p13
Campomelic dysplasia-1 (2)	**17q24.3-q25.1**
Carbamoylphosphate synthetase I deficiency (1)	2p
[Carbonic anhydrase I deficiency] (1)	8q22
Carboxypeptidase B deficiency (1)	**Chr.13**
?Cardiomyopathy (1)	2q35
Cardiomyopathy, dilated, X-linked (1)	Xp21.2
Cardiomyopathy, familial hypertrophic, 1, 192600 (3)	**14q12**
Cardiomyopathy, familial hypertrophic, 2 (2)	**1q3**
Cardiomyopathy, familial hypertrophic, 3 (2)	15q2
Cartilage-hair hypoplasia (2)	**9p13-q11**
Cat-eye syndrome (2)	22q11
?Cataract, anterior polar, I (2)	**14q24-qter**
?Cataract, congenital total (2)	Xp
Cataract, congenital, with microphthalmia (2)	**16p13.3**
Cataract, Coppock-like (3)	2q33-q35
Cataract, Marner type (2)	16q22.1
Cataract, zonular pulverulent-1 (2)	1q2
CD3, zeta chain, deficiency (1)	*1q23-q2521.1*
Central core disease (3)	**14q12**
Central core disease of muscle (2)	*19q13.1*
Centrocytic lymphoma (2)	11q13
Cerebral amyloid angiopathy (1)	20p11
Cerebral arteriopathy with subcortical infarcts and leukoencephalopathy (2)	**19q12**
Cerebrotendinous xanthomatosis (2)	2q33-qter
Ceroid lipofuscinosis, neuronal-1, infantile (2)	1p32
Cervical carcinoma (2)	11q13
[CETP deficiency] (1)	16q21
Charcot-Marie-Tooth neuropathy, slow nerve conduction type Ia (2)	17p11.2
Charcot-Marie-Tooth neuropathy, slow nerve conduction type Ib (2)	1q21.2-q23
Charcot-Marie-Tooth neuropathy, X-linked-1, dominant (2)	Xq13
Charcot-Marie-Tooth neuropathy, X-linked-2, recessive (2)	Xp22.2
Cholesteryl ester storage disease (1)	10q24-q25
?Chondrodysplasia punctata, rhizomelic (2)	**4p16-p14**
Chondrodysplasia punctata, X-linked dominant (2)	Xq28
Chondrodysplasia punctata, X-linked recessive (2)	Xp22.3
Choroideremia (2)	Xq21.2
Chronic granulomatous disease, autosomal, due to deficiency of CYBA (3)	16q24
Chronic granulomatous disease due to deficiency of NCF-1 (1)	7q11.23
Chronic granulomatous disease due to deficiency of NCF-2 (1)	1q25
Chronic granulomatous disease, X-linked (3)	Xp21.1
[Chronic infections, due to opsonin defect] (1)	10q11.2-q21
Citrullinemia (1)	9q34
Cleft palate, X-linked (2)	*Xq13-q21.31*
?Cleidocranial dysplasia (2)	**6q22**
CMO II deficiency (1)	8q21
Cockayne syndrome-2, late onset, 216410 (2)	**10q11**
Coffin-Lowry syndrome (2)	Xp22.2-p22.1
Colon cancer, familial, nonpolyposis type 1 (2)	**2p16-p15**
Colorblindness, blue monochromatic (3)	Xq28
Colorblindness, deutan (3)	Xq28
Colorblindness, protan (3)	Xq28
Colorblindness, tritan (2)	7q22-qter
Colorectal adenoma (1)	12p12.1
Colorectal cancer (1)	12p12.1
Colorectal cancer (1)	18q23.3
Colorectal cancer (1)	5q21
Colorectal cancer, 114500 (3)	17p13.1
Colorectal cancer (3)	*5q21-q22*
Combined C6/C7 deficiency (1)	5p13
?Combined variable hypogammaglobulinemia (1)	14q32.33
Congenital bilateral absence of vas deferens (1)	**7q31.2**
?Conotruncal cardiac anomalies (2)	**22q11**
Contractural arachnodactyly, congenital (3)	Chr.5
Coproporphyria (1)	Chr.9
?Cornelia de Lange syndrome (2)	3q26.3
[Coronary artery disease, susceptibility to] (1)	6q27
Cortisol resistance (1)	5q31
CR1 deficiency (1)	1q32
?Craniofrontonasal dysplasia (2)	**Xpter-p22.2**
Craniosynostosis, type II (2)	**6q24-qter**
Craniosynostosis, type I (2)	7p21.3-p21.2
[Creatine kinase, brain type, ectopic expression of] (2)	14q32
Creutzfeldt-Jakob disease, 123400 (3)	20pter-p12
Crigler-Najjar syndrome, type I, 218800 (1)	*Chr.2*
?Cryptorchidism (2)	Xp21
?Cutis laxa, marfanoid neonatal type (1)	7q31.1-q31.3
[Cystathion· inuria] (1)	Chr.16
Cystic fibrosis (3)	*7q31.2*
?Cystinuria (2)	**14q22**
?Cystinuria, 220100 (1)	**2pter-q22.3**
Deafness, conductive, with stapes fixation (2)	Xq13-q21.1
Deafness, low-tone (2)	6q21-q33
Debrisoquine sensitivity (1)	*22q13.1*
Dentinogenesis imperfecta-1 (2)	4q13-q21
Denys-Drash syndrome (1)	11p13
Diabetes insipidus, nephrogenic (3)	Xq28
Diabetes insipidus, neurohypophyseal, 125700 (1)	*20p13*
?Diabetes mellitus, insulin-dependent-1 (2)	6p21.3
Diabetes mellitus, insulin-resistant, with acanthosis nigricans (1)	*19p13.2*
Diabetes mellitus, rare form (1)	11p15.5
Diastrophic dysplasia (2)	5q31-q34
DiGeorge syndrome (2)	22q11
Diphenylhydantoin toxicity (1)	1p11-qter
[Diphtheria, susceptibility to] (1)	5q23
DNA ligase I deficiency (1)	**19q13.2-q13.3**
?Dubin-Johnson syndrome (2)	13q34
Duchenne muscular dystrophy (3)	Xp21.2
[Dysalbuminemic hyperthyroxinemia] (1)	4q11-q13
[Dysalbuminemic hyperzincemia] (1)	4q11-q13
Dysautonomia, familial (2)	**9q31-q33**
Dysfibrinogenemia, alpha types (1)	4q28
Dysfibrinogenemia, beta types (1)	4q28
Dysfibrinogenemia, gamma types (1)	4q28
Dyskeratosis congenita (2)	Xq28
?Dyslexia-1 (2)	15q11
Dysplasminogenemic thrombophilia (1)	6q26-q27
Dysprothrombinemia (1)	11p11-q12
[Dystransthyretinemic hyperthyroxinemia] (1)	18q11.2-q12.1
?EEC syndrome (2)	7q11.2-q21.3
Ehlers-Danlos syndrome, type IV, 130050 (3)	2q31
Ehlers-Danlos syndrome, type VI, 225400 (1)	1p36.3-p36.2
Ehlers-Danlos syndrome, type VIIA1, 130060 (3)	17q21.31-q22.05
Ehlers-Danlos syndrome, type VIIA2, 130060 (3)	*7q22.1*
?Ehlers-Danlos syndrome, type X (1)	*2q34*
[Elliptocytosis, Malaysian-Melanesian type] (1)	17q21-q22
Elliptocytosis-1 (3)	1p36.2-p34
Elliptocytosis-2 (2)	1q21
Elliptocytosis-3 (2)	14q22-q23.2
Emery-Dreifuss muscular dystrophy (2)	Xq28
Emphysema (1)	14q32.1
Emphysema due to alpha-2-macroglobulin deficiency (1)	12p13.3-p12.3

Disorder	Location	Disorder	Location
Emphysema-cirrhosis (1)	14q32.1	Glaucoma, congenital (2)	Chr.11
Endocardial fibroelastosis-2 (2)	Xq28	Glaucoma, primary open angle (2)	1q21-q31
Enolase deficiency (1)	1pter-p36.13	Glioblastoma multiforme (2)	10p12-q23.2
?Eosinophilic myeloproliferative disorder (2)	12p13	Glucose/galactose malabsorption (1)	22q11.2-qter
Epidermolysis bullosa dystrophica, dominant, 131750 (3)	*3p21.3*	Glutaricacidemia type IIC (3)	4q32-qter
Epidermolysis bullosa dystrophica, recessive, 226600 (3)	3p21.3	Glutaricaciduria, type IIA (1)	15q23-q25
Epidermolysis bullosa, Ogna type (2)	8q24	Glutaricaciduria, type IIB (2)	Chr.19
Epidermolysis bullosa simplex, 131900 (3)	17q12-q21	Glutathioninuria (1)	22q11.1-q11.2
Epidermolysis bullosa simplex, Dowling-Meara type, 131760 (3)	12q11-q13	Glycerol kinase deficiency (2)	Xp21.3-p21.2
Epidermolysis bullosa simplex, Dowling-Meara type, 131760 (3)	17q12-q21	Glycogen storage disease III (3)	1p21
Epidermolysis bullosa simplex, generalized, 131900 (1)	*12q11-q13*	Glycogen storage disease VII (1)	1cen-q32
?Epidermolysis bullosa simplex, localized, 131800 (1)	12q11-q13	Glycogen storage disease, X-linked hepatic (2)	Xp22.2-p22.1
?Epidermolysis bullosa, Weber Cockayne type, 131800 (2)	12q11-q13	[Glyoxalase II deficiency] (1)	16p13
Epidermolytic hyperkeratosis, 113800 (1)	17q21-q22	GM1-gangliosidosis (1)	3p21-p14.2
Epidermolytic hyperkeratosis, 113800 (3)	12q11-q13	GM2-gangliosidosis, AB variant (1)	Chr.5
Epidermolytic palmoplantar keratoderma (2)	17q11-q23	GM2-gangliosidosis, juvenile, adult (1)	15q23-q24
Epilepsy, benign neonatal (2)	20q13.2-q13.3	Goeminne TKCR syndrome (2)	Xq28
Epilepsy, juvenile myoclonic (2)	6p21.3	Goiter, adolescent, multinodular (1)	8q24.2-q24.3
Epilepsy, progressive myoclonic (2)	21q22.3	Goiter, nonendemic, simple (1)	8q24.2-q24.3
Epithelioma, self-healing, squamous 1, Ferguson-Smith type (2)	9q31	?Goldenhar syndrome (2)	7p
?Erythremia (1)	7q21	Gonadal dysgenesis, XY female type (2)	Xp22-p21
Erythremias, alpha- (1)	16pter-p13.3	Gonadal dysgenesis, XY type (1)	Yp11.2
Erythremias, beta- (1)	11p15.5	Gonadoblastoma (2)	11p13
Erythroblastosis fetalis (1)	1p36.2-p34	?Gonadotropin deficiency (2)	Xp21
[Erythrocytosis, familial], 133100 (2)	19p13.3-p13.2	Greig craniopolysyndactyly syndrome (3)	7p13
Erythrokeratodermia variabilis (2)	1p36.2-p34	?Gynecomastia, familial, due to increased aromatase activity (1)	15q21.1
[Euthyroidal hyper- and hypothyroxinemia] (1)	*Xq22*	Gyrate atrophy of choroid and retina with ornithinemia, B6 responsive or unresponsive (1)	10q26
Ewing sarcoma (3)	22q12	Harderoporphyrinuria (1)	Chr.9
Exertional myoglobinuria due to deficiency of LDH-A (1)	11p15.4	?Hartnup disease, 234500 (1)	2pter-q32.3
Exudative vitreoretinopathy, X-linked (2)	Xq21.31	Heinz body anemias, alpha- (1)	16pter-p13.3
Fabry disease (3)	Xq22	Heinz body anemias, beta- (1)	11p15.5
Facioscapulohumeral muscular dystrophy (2)	*4q35*	Hemochromatosis (2)	6p21.3
Factor H deficiency (1)	1q32	Hemodialysis-related amyloidosis (1)	15q21-q22
Factor V deficiency (1)	1q23	Hemolytic anemia due to ADA excess (1)	20q13.11
Factor VII deficiency (1)	13q34	Hemolytic anemia due to adenylate kinase deficiency (1)	9q34.1
Factor X deficiency (1)	13q34	Hemolytic anemia due to bisphosphoglycerate mutase deficiency (1)	7q31-q34
Factor XI deficiency (1)	4q35	Hemolytic anemia due to G6PD deficiency (1)	Xq28
Factor XII deficiency (1)	5q33-qter	*Hemolytic anemia due to glucosephosphate isomerase deficiency (1)*	*19q13.1*
Factor XIIIA deficiency (3)	6p25-p24	Hemolytic anemia due to glutathione peroxidase deficiency (1)	3q11-q12
Factor XIIIB deficiency (1)	1q31-q32.1	Hemolytic anemia due to glutathione reductase deficiency (1)	8p21.1
Familial Mediterranean fever (2)	*16p13*	Hemolytic anemia due to hexokinase deficiency (1)	10q22
?Fanconi anemia (1)	*1q42*	Hemolytic anemia due to PGK deficiency (1)	Xq13
Fanconi anemia-1 (2)	*20q13.2-q13.3*	Hemolytic anemia due to phosphofructokinase deficiency (1)	21q22.3
Favism (1)	Xq28	Hemolytic anemia due to triosephosphate isomerase deficiency (1)	12p13
[?Fetal alcohol syndrome] (1)	12q24.2	Hemophilia A (3)	Xq28
?Fetal hydantoin syndrome (1)	1p11-qter	Hemophilia B (3)	Xq27.1-q27.2
?Fibrodysplasia ossificans progressiva (1)	*20p13*	Hemorrhagic diathesis due to "antithrombin" Pittsburgh (1)	14q32.1
Fish-eye disease (3)	16q22.1	Hemorrhagic diathesis due to PAI1 deficiency (1)	7q21.3-q22
[Fish-odor syndrome] (1)	1q	?Hepatic lipase deficiency (1)	15q21-q23
Fletcher factor deficiency (1)	4q35	?Hepatocarcinoma (1)	2q14-q21
Focal dermal hypoplasia (2)	Xp22.31	Hepatocellular carcinoma (3)	4q32.1
Friedreich ataxia (2)	9q13-q21.1	[Hereditary persistence of alpha-fetoprotein] (3)	4q11-q13
Fructose intolerance (1)	9q22	?Hereditary persistence of fetal hemoglobin (3)	11p15.5
Fucosidosis (1)	1p34	?Hereditary persistence of fetal hemoglobin, heterocellular, Indian type (2)	7q36
Fumarase deficiency (1)	1q42.1	?Hereditary persistence of fetal hemoglobin, Swiss type (2)	Xp11.23
G6PD deficiency (3)	Xq28	?Hermansky-Pudlak syndrome, 203300 (1)	15q15
?Galactokinase deficiency (1)	17q21-q22	Hers disease, or glycogen storage disease VI (1)	Chr.14
Galactose epimerase deficiency (1)	1p36-p35	Heterocellular hereditary persistence of fetal hemoglobin (2)	11p15
Galactosemia (1)	9p13	[Hex A pseudodeficiency] (1)	15q23-q24
Galactosialidosis (1)	20q13.1	?HHH syndrome (2)	13q34
Gardner syndrome (3)	*5q21-q22*	[Histidinemia] (1)	12q22-q23
Gaucher disease (1)	1q21	Holoprosencephaly, type 2 (2)	7q36
Gaucher disease, variant form (1)	10q21-q22	?Holoprosencephaly-1 (2)	18pter-q11
Genitourinary dysplasia (2)	11p13		
Gerstmann-Straussler disease, 137440 (3)	20pter-p12		
?Gilbert syndrome, 143500 (1)	*Chr.2*		
Glanzmann thrombasthenia, type A (1)	17q21.32		
Glanzmann thrombasthenia, type B (1)	17q21.32		

Disorder	Location
?Holoprosencephaly-2 (2)	2p21
?Holoprosencephaly-4 (2)	14q11.1-q13
?Holt-Oram syndrome (2)	14q23-q24.2
?Holt-Oram syndrome (2)	20p13
Homocystinuria, B6-responsive and nonresponsive types (1)	21q22.3
HPFH, deletion type (1)	11p15.5
HPFH, nondeletion type A (1)	11p15.5
HPFH, nondeletion type G (1)	11p15.5
HPRT-related gout (1)	Xq26-q27.2
?Humoral hypercalcemia of malignancy (1)	12p12.1-p11.2
Huntington disease (2)	4p16.3
Hurler syndrome (1)	4p16.3
Hurler-Scheie syndrome (1)	4p16.3
Hydrocephalus due to aqueduct of Sylvius, 307000 (3)	Xq28
Hydrops fetalis, one form (1)	*19q13.1*
Hyperammonemia due to GTPase deficiency (1)	*1p13-p11*
Hyperbetalipoproteinemia (1)	2p24
Hypercalcemia, hypocalciuric, familial (2)	3q21-q24
Hypercholesterolemia, familial (3)	19p13.2-p13.1
?Hyperglycinemia, isolated nonketotic, type I (2)	9p22
?Hyperimmunoglobulin G1 syndrome (2)	14q32.33
Hyperkalemic periodic paralysis (3)	17q23.1-q25.3
?Hyperleucinemia-isoleucinemia or hypervalinemia (1)	12pter-q12
Hyperlipoproteinemia I (1)	8p22
Hyperlipoproteinemia, type Ib (1)	*19q13.2*
Hyperlipoproteinemia, type III (1)	19q13.2
[Hyperphenylalaninemia, mild] (3)	12q24.1
[?Hyperproglucagonemia] (1)	2q36-q37
Hyperproinsulinemia, familial (1)	11p15.5
?Hypertension, essential, 145500 (1)	17q21-q22
[Hypertension, essential, susceptibility to] (3)	1q42-q43
Hypertriglyceridemia, one form (1)	11q23
?Hypervalinemia or hyperleucine-isoleucinemia (1)	Chr.19
Hypoalphalipoproteinemia (1)	11q23
Hypobetalipoproteinemia (1)	2p24
Hypocalciuric hypercalcemia, type II (2)	19p13.3
[Hypoceruloplasminemia, hereditary] (1)	3q21-q24
Hypofibrinogenemia, gamma types (1)	4q28
?Hypoglycemia due to PCK1 deficiency (1)	20q13.31
Hypogonadism, hypergonadotropic (1)	19q13.32
?Hypogonadism, hypogonadotropic due to GNRH deficiency, 227200 (1)	8p21-p11.2
Hypomagnesemia, X-linked primary (2)	Xp22
?Hypomelanosis of Ito (2)	15q11-q13
?Hypomelanosis of Ito (2)	9q33-qter
Hypoparathyroidism, familial (1)	11p15.3-p15.1
Hypoparathyroidism, X-linked (2)	Xq26-q27
?Hypophosphatasia, adult, 146300 (1)	1p36.1-p34
Hypophosphatasia, infantile, 241500 (3)	1p36.1-p34
Hypophosphatemia, hereditary (2)	Xp22.2-p22.1
?Hypophosphatemia with deafness (2)	Xp22
Hypoprothrombinemia (1)	11p11-q12
?Hypospadias-dysphagia syndrome (2)	5p13-p12
Hypothyroidism, hereditary congenital (1)	8q24.3-q24.3
Hypothyroidism, nongoitrous (1)	1p13
Hypothyroidism, nongoitrous, due to TSH resistance (1)	14q31
I ?Ichthyosis vulgaris, 146700 (1)	1q21
Ichthyosis, X-linked (3)	Xp22.32
?Immotile cilia syndrome (2)	6p
Immunodeficiency, X-linked, with hyper-IgM (3)	Xq24-q27
Incontinentia pigmenti, familial (2)	Xq27-q28
Incontinentia pigmenti, sporadic type (2)	Xp11.21
Infertile male syndrome (1)	Xcen-q22
[Inosine triphosphatase deficiency] (1)	20p
Insomnia, fatal familial (3)	20pter-p12
?Insulin-dependent diabetes mellitus-2 (2)	11q
Interferon, alpha, deficiency (1)	9p21
Interferon, immune, deficiency (1)	12q24.1
?Isolated growth hormone deficiency due to defect in GHRF (1)	20p11.23-qter
Isolated growth hormone deficiency, IIIig type with absent GH and Kowarski type with bioinactive GH (3)	17q22-q24
Isovalericacidemia (1)	15q14-q15
J ?Jacobsen syndrome (2)	11q
K Kallmann syndrome (2)	Xp22.3
[Kappa light chain deficiency] (1)	2p12
Keratosis follicularis spinulosa decalvans (2)	Xp22.2-p21.2
[Kininogen deficiency] (1)	3q26-qter
?Klippel-Feil syndrome (2)	8q11.2
Kniest dysplasia (1)	12q13.11-q13.2
?Kostmann agranulocytosis (2)	6p21.3
Krabbe disease (1)	*14q31.3-q32.1*
L ?Lactase deficiency, adult, 223100 (1)	Chr.2
?Lactase deficiency, congenital (1)	Chr.2
?Lactoferrin-deficient neutrophils, 245480 (1)	3q21-q23
Langer-Giedion syndrome (2)	8q24.11-q24.13
Langer-Saldino achondrogenesis-hypochondrogenesis (1)	12q13.11-q13.2
Laron dwarfism (1)	5p13-p12
?Laryngeal adductor paralysis (2)	6p21.3-p21.2
[Lead poisoning, susceptibility to] (1)	9q34
?Leiomyomata, multiple hereditary cutaneous (2)	18p11.32
Leiomyomatosis-nephropathy syndrome, 308940 (1)	Xq22
Leprechaunism (1)	*19p13.2*
Lesch-Nyhan syndrome (3)	Xq26-q27.2
?Letterer-Siwe disease (2)	13q14-q31
Leukemia, acute lymphoblastic (1)	*19p13.3*
Leukemia, acute lymphoblastic (2)	9p22-p21
?Leukemia, acute lymphocytic, with 4/11 translocation (3)	4q21
Leukemia, acute myeloid (3)	*21q22.3*
Leukemia, acute myeloid, M2 type (1)	*Xp22.32*
Leukemia, acute pre-B-cell (2)	1q23
Leukemia, acute promyelocytic (1)	17q21.1
Leukemia, acute promyelocytic (2)	15q22
Leukemia, acute, T-cell (2)	11p13
Leukemia, chronic myeloid (3)	22q11.21
Leukemia, chronic myeloid (3)	9q34.1
Leukemia, multilineage (2)	Chr.4
Leukemia, myeloid/lymphoid or mixed-lineage (2)	11q23
Leukemia, T-cell acute lymphoblastic (2)	11p15
Leukemia, T-cell acute lymphoblastic (2)	9q34.3
Leukemia, T-cell acute lymphoblastoid (2)	*19p13.2-p13.1*
Leukemia, T-cell acute lymphocytic (2)	10q24
?Leukemia, transient (2)	21q11.2
Leukemia-1, T-cell acute lymphoblastic (3)	1p32
Leukemia-2, T-cell acute lymphoblastic (3)	9q31
Leukemia/lymphoma, B-cell, 1 (2)	11q13.3
Leukemia/lymphoma, B-cell, 2 (2)	18q21.3
Leukemia/lymphoma, B-cell, 3 (2)	19q13
Leukemia/lymphoma, T-cell (2)	14q32.1
Leukemia/lymphoma, T-cell (2)	2q34
Leukemia/lymphoma, T-cell (3)	14q11.2
Leukocyte adhesion deficiency (1)	21q22.3
Li-Fraumeni syndrome (1)	17p13.1
Lipoamide dehydrogenase deficiency (1)	7q31-q32
Lipoma (2)	12q13-q14
Liver cell carcinoma (1)	11p14-p13
Long QT syndrome (2)	11p15.5
Lowe syndrome (3)	Xq26.1
Lupus erythematosus, systemic, 152700 (1)	1q23
Lymphoproliferative syndrome, X-linked (2)	Xq25
?Lynch cancer family syndrome II (2)	18q11-q12
?Lysosomal acid phosphatase deficiency (1)	11p12-p11
M Macrocytic anemia of 5q-syndrome, refractory (2)	5q12-q32
Macrocytic anemia refractory, of 5q-syndrome, 153550 (3)	5q31.1
Macular dystrophy (1)	6p21.1-cen
Macular dystrophy, atypical vitelliform (2)	8q24
Macular dystrophy, North Carolina type (2)	6q14-q16.2
Macular dystrophy, vitelliform type (2)	11q13

Disorder	Location
?Male infertility due to acrosin deficiency (2)	22q13-qter
?Male infertility, familial (1)	11p13
?Male pseudohermaphroditism due to defective LH (1)	19q13.32
Malignant hyperthermia susceptibility-1, 145600 (3)	19q13.1
Malignant hyperthermia susceptibility-2, 145600 (2)	**17q11.2-q24**
Malignant melanoma, cutaneous (2)	1p36
?Manic-depressive illness, X-linked (2)	Xq28
Mannosidosis (1)	19p13.2-q12
Maple syrup urine disease, type 1 (3)	19q13.1-q13.2
Maple syrup urine disease, type 2 (3)	1p31
Maple syrup urine disease, type 3 (1)	6p22-p21
Marfan syndrome, 154700 (3)	15q21.1
Maroteaux-Lamy syndrome, several forms (1)	5q11-q13
Martin-Bell syndrome (2)	Xq27.3
MASA syndrome (2)	Xq28
McArdle disease (1)	11q13
McCune-Albright polyostotic fibrous dysplasia, 174800 (1)	20q13.2
[McLeod phenotype] (2)	Xp21.2-p21.1
Medullary thyroid carcinoma (2)	*10q11.2*
Megacolon (2)	10q11.2
Megalocornea, X-linked (2)	Xq21.3-q22
Melanoma, cutaneous malignant (2)	**9p21**
Meningioma (2)	22q12.3-qter
Meningioma (3)	22q12.3-q13.1
Menkes disease (2)	Xq12-q13
Mental retardation of WAGR (2)	11p13
Mental retardation, Snyder-Robinson type (2)	**Xp21**
?Mental retardation, X-linked nonspecific, with aphasia (2)	**Xp11**
Mental retardation, X-linked, syndromic-1, with dystonic movements, ataxia, and seizures (2)	Xp22.2-p22.1
Mental retardation, X-linked, syndromic-2, with dysmorphism and cerebral atrophy (2)	Xp11-q21
Mental retardation, X-linked, syndromic-3, with spastic diplegia (2)	Xp11-q21.3
Mental retardation, X-linked, syndromic-4, with congenital contractures and low fingertip arches (2)	Xq13-q22
Mental retardation, X-linked, syndromic-6, with Dandy-Walker malformation, basal ganglia disease, and seizures (2)	Xq25-q27
Mental retardation, X-linked, syndromic-6, with gynecomastia and obesity (2)	Xp21.1-q22
Mental retardation, X-linked-1, non-dysmorphic (2)	Xp22
?Mental retardation, X-linked-2, non-dysmorphic (2)	Xq11-q12
Mental retardation, X-linked-3 (2)	Xq28
Mental retardation–skeletal dysplasia (2)	Xq28
Metachromatic leukodystrophy (1)	22q13.31-qter
Metachromatic leukodystrophy due to deficiency of SAP-1 (1)	10q21-q22
Methemoglobinemia due to cytochrome b5 deficiency (3)	Chr.18
Methemoglobinemia, enzymopathic (1)	22q13.31-qter
Methemoglobinemias, alpha- (1)	16pter-p13.3
Methemoglobinemias, beta- (1)	11p15.5
Methylmalonicaciduria, mutase deficiency type (1)	6p21
Mevalonicaciduria (1)	**Chr.12**
?Microphthalmia with linear skin defects (2)	Xp22.3
Miller-Dieker lissencephaly syndrome (2)	17p13.3
?Mitochondrial complex I deficiency, 252010 (1)	**11q13**
MODY, one form (3)	11p15.5
MODY, type I (2)	20q13
MODY, type IL, 125851 (3)	7p15-p13
?Moebius syndrome (2)	13q12.2-q13
?Monocyte carboxyesterase deficiency (1)	16q13-q22.1
Mucolipidosis II (1)	4q21-q23
Mucolipidosis III (1)	4q21-q23
Mucopolysaccharidosis I (1)	4p16.3
Mucopolysaccharidosis II (2)	Xq28
Mucopolysaccharidosis IVA (3)	**16q24.3**
Mucopolysaccharidosis IVB (1)	3p21-p14.2
Mucopolysaccharidosis VII (1)	7q21.11
Multiple endocrine neoplasia I (1)	11q13
Multiple endocrine neoplasia II (2)	*10q11.2*
Multiple endocrine neoplasia III (2)	*10q11.5*
?Multiple exostoses (2)	8q23-q24.1
?Multiple lipomatosis (2)	12q13-q14
[Multiple sclerosis, susceptibility to] (2)	**18q22-qter**
?Muscle glycogenosis (1)	Xq12-q13
Muscular dystrophy, Duchenne-like, autosomal (2)	**18q12-q18**
Muscular dystrophy, limb-girdle, autosomal dominant (2)	*5q22.3-q31.3*
Muscular dystrophy, limb-girdle, autosomal recessive (2)	15q15-q22
Myelodysplastic syndrome, preleukemic (2)	**5q31.1**
Myelogenous leukemia, acute (3)	5q31.1
Myeloperoxidase deficiency (1)	17q21.3-q22
Myoadenylate deaminase deficiency (1)	1p21-p13
[Myocardial infarction, susceptibility to] (3)	**17q23**
Myoglobinuria/hemolysis due to PGK deficiency (1)	Xq13
Myopathy due to CTPase deficiency (1)	*1p13-p11*
Myopathy due to phosphoglycerate mutase deficiency (1)	7p13-p12.3
Myopia-1 (2)	Xq28
Myotonia congenita, atypical acetazolamide-responsive (2)	17q23.1-q25.3
Myotonia congenita, dominant, 160800 (2)	**7q35**
Myotonia congenita, recessive, 255700 (3)	*7q35*
Myotonic dystrophy (2)	19q13.2-q13.3
Myotubular myopathy, X-linked (2)	Xq28
Myxoid liposarcoma (2)	12q13-q14
?N syndrome, 310465 (1)	Xp22.3-p21.1
Nail-patella syndrome (2)	9q34
Nance-Horan syndrome (2)	Xp22.3-p21.1
Nemaline myopathy-1 (2)	1q21-q23
Nephronophthisis, juvenile (2)	**2p23-cen**
Neuroblastoma (2)	1p36.2-p36.1
Neuroepithelioma, 133450 (1)	**11q23-q24**
Neuroepithelioma (2)	22q12
Neurofibromatosis, von Recklinghausen (3)	17q11.2
Neuropathy, recurrent, with pressure palsies, 162500 (3)	**17p11.2**
Neutropenia, immune (2)	1q23
Niemann-Pick disease, type A (1)	11p15.4-15.1
Niemann-Pick disease, type B (1)	11p15.4-15.1
Niemann-Pick disease, type C (2)	**18p**
Nightblindness, congenital stationary, type I (2)	Xp11.3
[Non-insulin dependent diabetes mellitus, susceptibility to] (2)	**19q13.3**
Norrie disease (2)	Xp11.4
Norum disease (3)	16q22.1
Nucleoside phosphorylase deficiency, immunodeficiency due to (1)	14q13.1
?Obesity (2)	7q31
?Ocular albinism autosomal recessive (2)	6q13-q15
Ocular albinism, Forsius-Eriksson type (2)	Xp11-q11
Ocular albinism, Nettleship-Falls type (2)	Xp22.3
Ornithine transcarbamylase deficiency (3)	Xp21.1
Orofacial cleft (2)	6pter-p23
Oroticaciduria (1)	3q13
Osteoarthrosis, precocious (3)	12q13.11-q13.2
Osteogenesis imperfecta, 4 clinical forms, 166200, 166210, 259420, 166220 (3)	*17q21.31-q22.05*
Osteogenesis imperfecta, 4 clinical forms, 166200, 166210, 259420, 166220 (3)	*7q22.1*
?Osteopetrosis, 259700 (1)	1p21-p13
Osteoporosis, idiopathic, 166710 (3)	**17q21.31-q22.05**
Osteosarcoma, 259500 (2)	**13q14.1-q14.2**
Otopalatodigital syndrome, type I (2)	Xq28
Ovarian carcinoma, 167000 (2)	**19q13.1-q13.2**
Ovarian carcinoma (2)	9p24
Ovarian failure, premature (2)	Xq26-q27
Oxalosis I (1)	2q36-q37
?Paget disease of bone (2)	6q21.3
?Pallister-Hall syndrome (2)	**3p25.3**
Pancreatic lipase deficiency (1)	10q26.1
?Panhypopituitarism (1)	3q

Disorder	Location	Disorder	Location
?Panhypopituitarism, X-linked (2)	Xq21.3-q22	**Retinitis pigmentosa, autosomal recessive (8)**	**8q21-q24**
Paraganglioma (2)	*11q22.3-q23.2*	**Retinitis pigmentosa, peripherin-related (8)**	**6p21.1-cen**
Paramyotonia congenita, 168300 (3)	17q23.1-q25.3	Retinitis pigmentosa-1 (2)	8p11-q21
Parathyroid adenomatosis 1 (2)	11q13	Retinitis pigmentosa-2 (2)	Xp11.3
?Parietal foramina (2)	**11p12-p11.12**	*Retinitis pigmentosa-3 (2)*	*Xp21.1*
[?Parkinsonism, susceptibility to] (1)	*22q13.1*	**Retinitis pigmentosa-4, autosomal dominant (8)**	**8q21-q24**
Paroxysmal nocturnal hemoglobinuria (1)	Xq22.1	**Retinitis pigmentosa-5 (2)**	**2q**
Pelizaeus-Merzbacher disease (3)	Xq22	?Retinitis pigmentosa-6 (2)	Xp21.3-p21.2
Pelviureteric junction obstruction (2)	**6p**	**Retinitis pigmentosa-9 (2)**	**7p15.1-p15**
?Pendred syndrome (2)	8q24	**Retinitis pigmentosa-10 (2)**	**7q**
Periodontitis, juvenile (2)	4q11-q13	**Retinitis punctata albescens (1)**	**6p21.1-cen**
Persistent Mullerian duct syndrome (1)	19p13.3-p13.2	Retinoblastoma (3)	13q14.1-q14.2
Phenylketonuria (3)	12q24.1	?Retinol binding protein, deficiency of (1)	10q23-q24
Phenylketonuria due to dihydropteridine reductase deficiency (1)	4p15.31	Retinoschisis (2)	Xp22.3-p22.1
Pheochromocytoma (2)	**1p**	?Retz syndrome (2)	Xp
Phosphoribosyl pyrophosphate synthetase-related gout (1)	Xq22-q24	Rhabdomyosarcoma (2)	11p15.5
?Phosphorylase kinase deficiency of liver and muscle, 261750 (2)	16q12-q13.1	Rhabdomyosarcoma, alveolar (2)	2q37
Piebaldism (3)	*4q12*	*Rhabdomyosarcoma, alveolar (3)*	*2q35*
Pituitary tumor, growth-hormone-secreting (1)	20q13.2	Rh-null disease (1)	1cen-q22
PK deficiency hemolytic anemia (1)	1q21	?Rh-null hemolytic anemia (1)	1p36.2-p34
[Placental lactogen deficiency] (1)	17q22-q24	Rickets, vitamin D–resistant (1)	12q12-q14
Placental steroid sulfatase deficiency (3)	Xp22.32	*Rieger syndrome (2)*	*4q25-q27*
Plasmin inhibitor deficiency (1)	17pter-p12	Rod monochromacy (2)	Chr.14
Plasminogen activator deficiency (1)	8p12	?Rothmund-Thomson syndrome (2)	Chr.8
Plasminogen deficiency, types I and II (1)	6q26-q27	Rubinstein-Taybi syndrome (2)	16p13.3
Plasminogen Tochigi disease (1)	6q26-q27	**?Russell-Silver syndrome (2)**	**17q25**
Platelet alphaAdelta storage pool deficiency (1)	*1q23-q25*	**Saethre-Chotzen syndrome (2)**	**7p**
[Polio, susceptibility to] (2)	*19q13.2-q13.3*	Salivary gland pleomorphic adenoma (2)	8q12
Polycystic kidney disease (2)	16p13.31-p13.12	Sandhoff disease (1)	5q13
Polycystic ovarian disease (1)	17q11-q12	**?Sanfilippo disease, type IIIC (2)**	**Chr.14**
Polyposis coli, familial (3)	*5q21-q22*	Sanfilippo syndrome D (1)	12q14
Pompe disease (1)	17q23	Sarcoma, synovial (2)	Xp11.2
Porphyria, acute hepatic (1)	9q34	Schele syndrome (1)	4p16.3
Porphyria, acute intermittent (1)	11q24.1-q24.2	?Schizophrenia (2)	5q11.2-q13.3
Porphyria, Chester type (2)	11q	**Schizophrenia, chronic (3)**	**21q21.3-q22.05**
Porphyria, congenital erythropoietic (1)	10q25.2-q26.3	**[?Schizophrenia, susceptibility to] (2)**	**3q13.3**
Porphyria cutanea tarda (1)	1p34	Sclerotylosis (2)	4q28-q31
Porphyria, hepatoerythropoietic (1)	1p34	**Severe combined immunodeficiency, 202500 (1)**	**10p15-p14**
Porphyria variegata (2)	14q32	Severe combined immunodeficiency due to ADA deficiency (1)	20q13.11
Postanesthetic apnea (1)	3q26.1-q26.2	Severe combined immunodeficiency due to IL2 deficiency (1)	4q26-q27
Prader-Willi syndrome (2)	15q11	*Severe combined immunodeficiency, HLA class II–negative type (1)*	*19p13.1*
[Pre-eclampsia, susceptibility to] (3)	**1q42-q43**	Severe combined immunodeficiency, X-linked, 300400 (3)	Xq13
Progressive cone dystrophy (2)	Xp21.1-p11.3	**Short stature (2)**	**Xpter-p22.32**
Prolidase deficiency (1)	19cen-q13.11	?Sialidosis (2)	6p21.3
Properdin deficiency, X-linked (3)	Xp11.4-p11.23	Sickle cell anemia (1)	11p15.5
Propionicacidemia, type I or pccA type (1)	13q32	**?Simpson-Golabi-Behmel syndrome (2)**	**Xcen-q21.3**
Propionicacidemia, type II or pccB type (1)	3q21-q22	*?Situs inversus viscerum (2)*	*14q22*
Protein C deficiency (1)	2q13-q14	?SLE (1)	1q32
Protein C inhibitor deficiency (2)	**14q32.1**	Small-cell cancer of lung (2)	3p23-p21
Protein S deficiency (1)	3p11.1-q11.2	?Smith-Lemli-Opitz syndrome (2)	7q34-qter
Protoporphyria, erythropoietic (1)	*18pter-p11.21*	Smith-Magenis syndrome (2)	17p11.2
Pseudohermaphroditism, male, with gynecomastia (1)	17q11-q12	Spastic paraplegia, X-linked, uncomplicated (2)	Xq21-q22
Pseudohypoaldosteronism (1)	4q31.1	**Spherocytosis, hereditary (3)**	**17q21-q22**
Pseudohypoparathyroidism, type Ia (1)	20q13.2	Spherocytosis, hereditary, Japanese type (1)	15q15
Pseudovaginal perineoscrotal hypospadias (1)	**Chr.2**	Spherocytosis, recessive (1)	1q21
Pseudo-vitamin D dependency rickets I (2)	12q14	Spherocytosis-1 (3)	14q22-q23.2
Pseudo-Zellweger syndrome (1)	3p23-p22	Spherocytosis-2 (3)	8p11.2
?Pyridoxine dependency with seizures (1)	2q31	Spinal and bulbar muscular atrophy of Kennedy, 313200 (3)	Xcen-q22
Pyropoikilocytosis (1)	1q21	Spinal muscular atrophy II (2)	5q12.2-q13.3
Pyruvate carboxylase deficiency (1)	11q	Spinal muscular atrophy III (2)	5q12.2-q13.3
Pyruvate dehydrogenase deficiency (1)	Xp22.2-p22.1	Spinocerebellar ataxia-1 (2)	6p21.3-p21.2
?Rabson-Mendenhall syndrome (1)	**19p13.2**	**Spinocerebellar atrophy II (2)**	**12q24**
?Ragweed sensitivity (2)	6p21.3	Split-hand/foot deformity, type 1 (2)	7q21.2-q21.3
Reifenstein syndrome (1)	Xcen-q22	**Split-hand/split-foot deformity, type 2 (2)**	**Xq26**
Renal cell carcinoma (2)	3p14.2	Spondyloepiphyseal dysplasia congenita (3)	12q13.11-q13.2
[Renal glucosuria] (2)	6p21.3	Spondyloepiphyseal dysplasia tarda (2)	Xp22
Renal tubular acidosis-osteopetrosis syndrome (1)	8q22	**Startle disease (2)**	**5q33-q35**
?Retinal cone dystrophy-1 (2)	6q25-q26		
?Retinal cone-rod dystrophy (2)	18q21-q22.2		
Retinitis pigmentosa, autosomal dominant (1)	*11p13*		

Disorder	Location
Stickler syndrome (3)	12q13.11-q13.2
Sucrose intolerance (1)	3q25-q26
Supravalvar aortic stenosis (3)	7q11.2
Tay-Sachs disease (1)	15q23-q24
Testicular feminization (1)	Xcen-q22
Thalassemias, alpha- (1)	16pter-p13.3
Thalassemias, beta- (1)	11p15.5
Thrombocytopenia, X-linked (2)	Xp21-p11
Thrombophilia due to elevated HRG (1)	3p14-qter
Thrombophilia due to excessive plasminogen activator inhibitor (1)	7q21.3-q22
Thrombophilia due to heparin cofactor II deficiency (1)	22q11
Thyroid hormone resistance, 274300, 188570 (3)	3p24.3
Thyroid iodine peroxidase deficiency (1)	*2p13*
Thyroid papillary carcinoma (1)	10q11-q12
Thyrotropin-releasing hormone deficiency (1)	Chr.3
Torsion dystonia (2)	9q32-q34
Torsion dystonia-parkinsonism, Filipino type (2)	*Xq12-q21.1*
?Tourette syndrome (2)	18q22.1
Transcobalamin II deficiency (1)	22q11.2-qter
[Transcortin deficiency] (1)	*14q32.1*
Treacher Collins mandibulofacial dysostosis (2)	*5q32-q33.1*
Trichorhinophalangeal syndrome, type I (2)	8q24.12
Trypsinogen deficiency (1)	7q32-qter
{?Tuberculosis, susceptibility to} (2)	2q
Tuberous sclerosis-1 (2)	9q33-q34
?Tuberous sclerosis-2 (2)	11q23
?Tuberous sclerosis-3 (2)	12q23.3
Tuberous sclerosis-4 (2)	16p13
Turner syndrome (1)	Xq13.1
Tyrosinemia, type I (1)	15q23-q25
Tyrosinemia, type II (1)	16q22.1-q22.3
Urate oxidase deficiency (1)	1p22
Urolithiasis, 2,8-dihydroxyadenine (1)	16q24
Usher syndrome, type 1A (2)	14q32
Usher syndrome, type 1B (2)	11q13.5
Usher syndrome, type 1C (2)	11p
Usher syndrome, type 2 (2)	1q32
van der Woude syndrome (2)	1q32
Velocardiofacial syndrome (2)	22q11
Vitreoretinopathy, exudative, familial (2)	11q13-q23
Vitreoretinopathy, neovascular inflammatory (2)	11q13
{Vivax malaria, susceptibility to} (1)	1q21-q22
von Hippel-Lindau syndrome (2)	3p26-p25
von Willebrand disease (1)	12pter-p12
Waardenburg syndrome, type I (3)	2q35
Waardenburg syndrome, type III, 48820 (3)	2q35
Waisman parkinsonism-mental retardation syndrome (2)	Xq28
Watson syndrome, 193520 (3)	17q11.2
Werdnig-Hoffmann disease (2)	5q12.2-q13.3
Werner syndrome (2)	8p12-p11
{Weruicke-Korsakoff syndrome, susceptibility to} (1)	3p14.3
Wieacker-Wolff syndrome (2)	Xq13-q21
?Williams-Beuren syndrome (2)	4q33-q35.1
Wilms tumor (2)	11p13
Wilms tumor, type 2 (2)	11p15.5
Wilson disease (2)	13q14-q21
Wiskott-Aldrich syndrome (2)	Xp11.3-p11.2
?Wolf-Hirschhorn syndrome, 194190 (3)	4p16.1
Wolf-Hirschhorn syndrome (2)	*4p16.3*
Wolman disease (1)	10q24-q25
Wrinkly skin syndrome (2)	2q32
?Xeroderma pigmentosum (1)	1q42
Xeroderma pigmentosum, group B (3)	2q21
Xeroderma pigmentosum, complementation group C (2)	Chr.5
Xeroderma pigmentosum, group D, 278730 (1)	19q13.2-q13.3
Xeroderma pigmentosum, type A (1)	9q34.1
?Xeroderma pigmentosum, type F (2)	Chr.15
Zellweger syndrome, type II (1)	1p22-p21
Zellweger syndrome-1 (2)	7q11.23

The purpose of the above description and examples is to illustrate some embodiments of the present invention without implying any limitation. It will be apparent to those of skill in the art that various modifications and variations may be made to the composition and method of the present invention without departing from the spirit or scope of the invention. All patents and publications cited herein are incorporated by reference in entirety.

WE CLAIM:

1. A method for inducing tolerance to an antigen (Ag) which is manifested by suppressing both humoral and cell-mediated immune responses, comprising administering an effective amount of an Ag(mPEG) conjugate for the induction of tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses.

2. The method of claim 1, wherein said Ag(mPEG) conjugate inhibits activation of cytolytic activity by primed $CD8^+$ CTL.

3. The method of claim 1, wherein said tolerance of the humoral immune response is induced in an isotype-pan-specific manner.

4. The method of claim 1, wherein the tolerance is mediated by Ag-specific $CD8^+$ suppressor T (Ts) cells.

5. The method of claim 1, wherein said conjugate suppresses IL-2 production by lymph node lymphocytes (LNL).

6. The method of claim 1, wherein said conjugate suppresses IL-2, IFN-γ and IL-4 lymphokine production.

7. The method of claim 6 wherein said method does not influence $CD4^+$ T cells to express the characteristics of their Th1 or Th2 phenotype.

8. The method of claim 1, wherein said Ag(mPEG) conjugate inhibits lymphokine production by primed $CD4^+$ Th cells.

9. The method of claim 1, wherein said Ag(mPEG) conjugate inhibits both arms of humoral and cell-mediated immune responses in vivo and said tolerance induced by Ag(mPEG) conjugates is Ag specific.

10. A method of obtaining passive transfer of suppression of an immune response comprising treating an animal with Ag(mPEG) conjugate and transferring lymphocytes from said animal to a syngeneic recipient animal, wherein said lymphocytes provide suppression of Ag-specific cytotoxic lymphocyte (CTL) activity in said recipient animal.

11. The method of claim 10, wherein said transfer inhibited humoral and cytolytic responses in recipients are mediated by Ts cells.

12. A method of treating a condition selected from the group consisting of allergies and autoimmune diseases by inducing tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses comprising administering an effective amount of Ag(mPEG) conjugate to induce tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses.

13. A method of preventing an immune rejection of organ transplants or transplants of DNA transfected cells comprising administering an effective amount of Ag(mPEG) conjugate which is xenogeneic or allogeneic to a patient in need of said organ transplant, to induce tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses prior to the administration of the (Ag).

14. A method of claim 13, wherein antibodies of all IgG subclasses are suppressed.

15. The method of claim 14, wherein IgG isotypes dependent upon Th1 and Th2 lymphokines are both inhibited by said Ag(mPEG) conjugates.

16. The method of claim 14, wherein lymphokines produced by $CD4^+$ Th cells are inhibited by said Ag(mPEG) conjugate.

17. The method of claim 16 wherein said lymphokines are selected from the group consisting of IL-2, IL-4 and IFN-γ.

18. A method of treating organ-specific autoimmune diseases in animal comprising administration of mPEG conjugates of autoantigens selected from the group consisting of collagen-induced arthritis by type II collagen and diabetes in NOD mice by insulin to induce tolerance to an antigen (Ag) in both humoral and cell-mediated immune responses prior to adminstration of said (Ag).

19. A method of conducting gene therapy comprising the step of

administering to a mammal an immunosuppressive effective amount of a tolerogenic conjugate consisting of a protein coupled to monomethoxypolyethylene glycol (mPEG) having a molecular weight of about 2,000-10,000 daltons, wherein administration of said tolerogenic conjugate is at least one day prior to administration of a gene therapy vector encoding a gene for a protein, wherein said protein is identical to said protein which is coupled to mPEG, and wherein said method results in the suppression of an immune response and in the development of tolerance to the protein expressed by said gene encoded by said gene therapy vector.

20. A method of conducting gene therapy comprising the steps of

a) administering to a mammal an immunosuppressive effective amount of a tolerogenic conjugate consisting of a protein conjugated to monomethoxypolyethylene glycol (mPEG) having a molecular weight of about 2,000 to 10,000 daltons, wherein administration of said tolerogenic conjugate is at least one day prior to administration of DNA, RNA or mRNA and encoding a protein administered for gene therapy, wherein the encoded protein is identical to said protein which is conjugated to mPEG, and wherein said method results in the suppression of an immune response and in the development of tolerance to encoded protein of said DNA, RNA or mRNA administered for gene therapy.

21. Method of conducting gene therapy according to claim 20, wherein mPEG conjugates of both a vector protein and protein administered for gene therapy are administered prior to conducting gene therapy with a gene therapy vector encoding a gene for a therapeutic protein.

22. Method of conducting gene therapy according to claim 21, wherein the vector protein and protein administered for gene therapy are conjugated together as a hybrid mPEG conjugate and administered prior to conducting gene therapy with a gene therapy vector encoding a gene for a therapeutic protein.

23. Method of conducting gene therapy according to claim 20, wherein said protein administered for gene therapy is blood factor VIII protein.

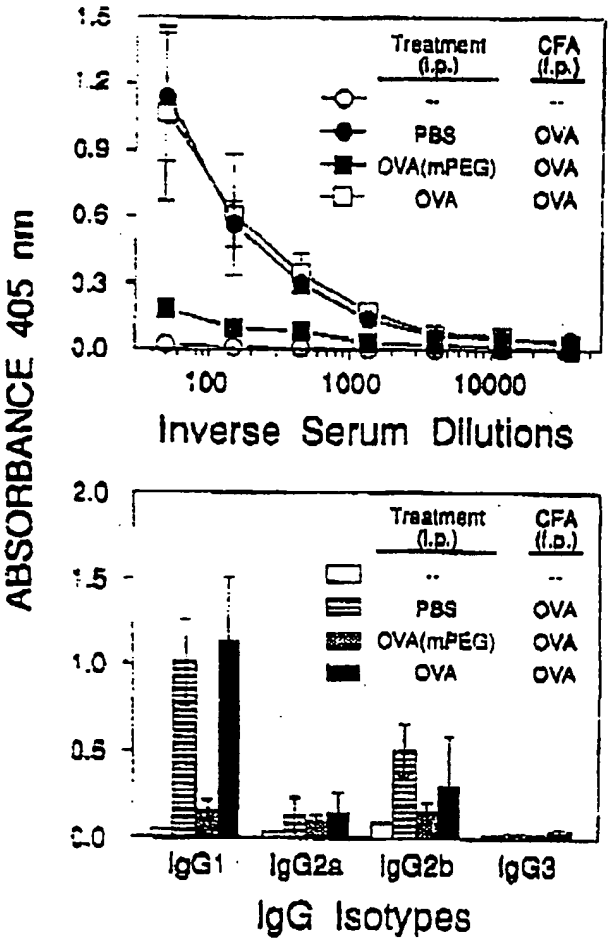
24. A method of treating hemophaelia comprising the steps of

administering an effective amount of a human blood factor protein(mPEG) conjugate for the induction of tolerance to said human blood clotting factor protein in both humoral and cell-mediated immune responses prior to administering a gene for said clotting factor encoded by a gene therapy vector.

25. A method according to claim 20 wherein said human blood factor is selected from the group consisting of human clotting blood factor VIII and human blood factor IV.

26. A method of overcoming the immunogenicity of a gene therapy vector, comprising administering an mPEG conjugate corresponding to a vector protein administered prior to the administration of the gene therapy vector.

27. A method according to claim 24 wherein the vector protein is a vector coat protein.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1.5
0.9
0.6
0.3
0.0
100
1000
10000
Inverse Serum Dilutions
Treatment (i.p.)
CFA (i.p.)
--
--
PBS
OVA
OVA(mPEG)
OVA
OVA
OVA
2.0
1.5
1.0
0.5
0.0
IgG1
IgG2a
IgG2b
IgG3
IgG Isotypes

Figure 1

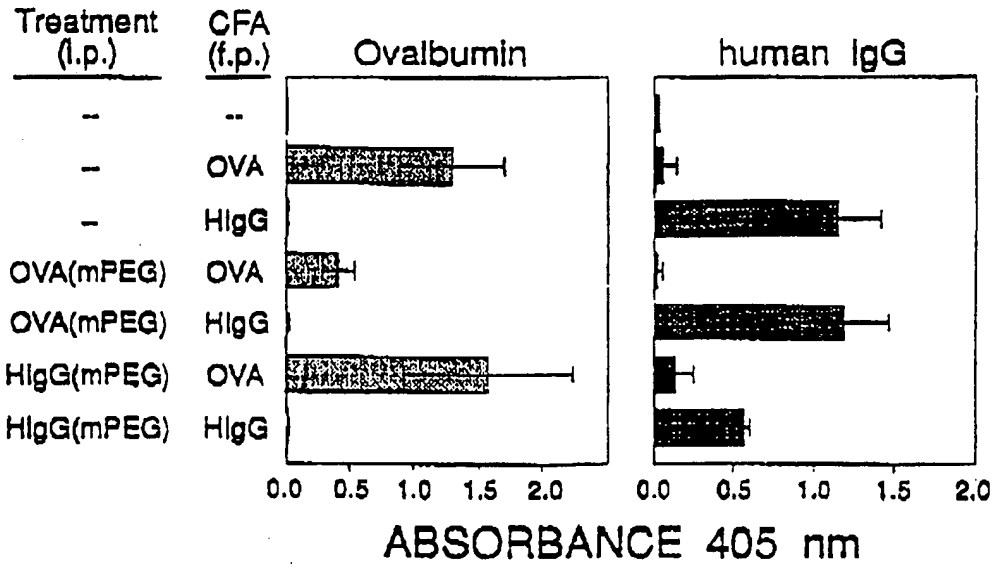

Treatment (I.p.)
CFA (f.p.)
Ovalbumin
human IgG
--
--
OVA
HIgG
OVA(mPEG)
OVA
HIgG
HIgG(mPEG)
OVA
HIgG
0.0 0.5 1.0 1.5 2.0
0.0 0.5 1.0 1.5 2.0
ABSORBANCE 405 nm

Figure 2

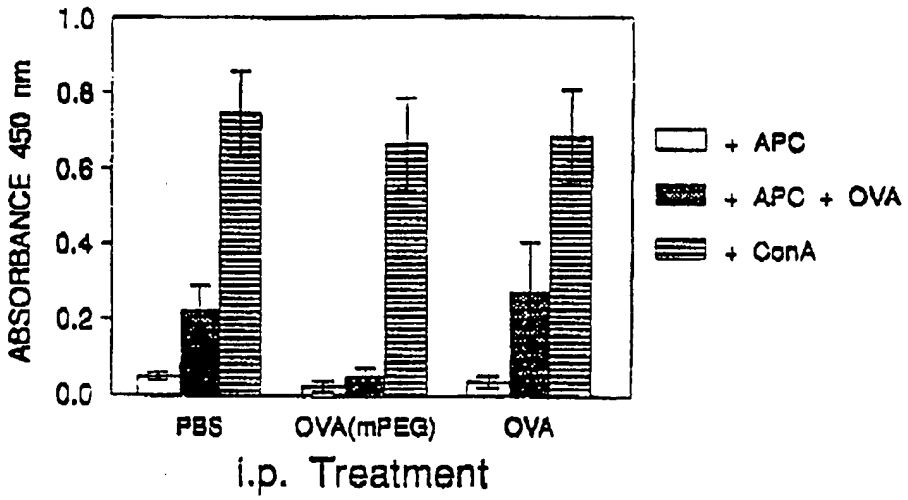

1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE 450 nm
PBS
OVA(mPEG)
OVA
i.p. Treatment
+ APC
+ APC + OVA
+ ConA

Figure 3

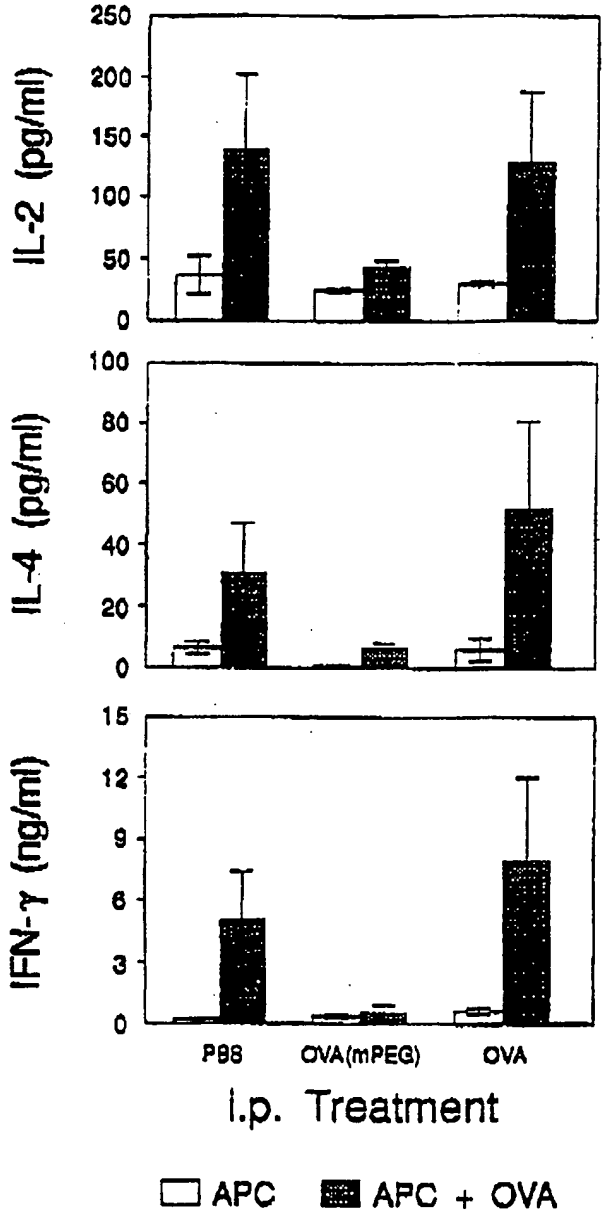

IL-2 (pg/ml)
250
200
150
100
50
0
IL-4 (pg/ml)
100
80
60
40
20
0
IFN-γ (ng/ml)
15
12
9
6
3
0
P88
OVA(mPEG)
OVA
I.p. Treatment
APC
APC + OVA

Figure 4

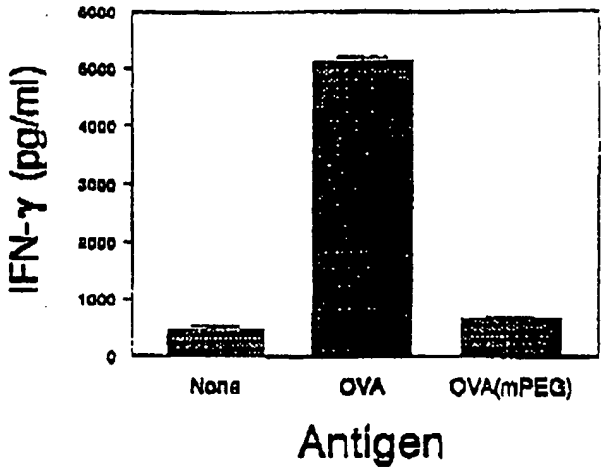

IFN-γ (pg/ml)
6000
5000
4000
3000
2000
1000
0
None
OVA
OVA(mPEG)
Antigen

Figure 5

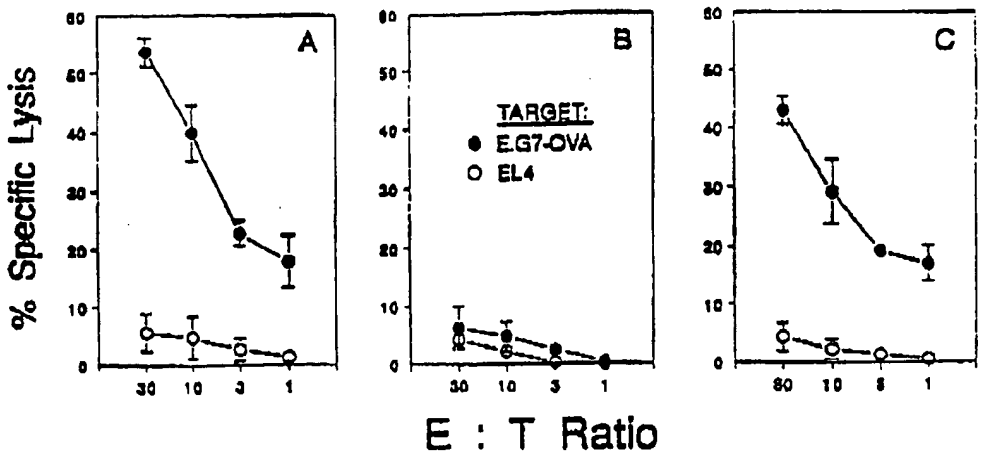


Figure 6

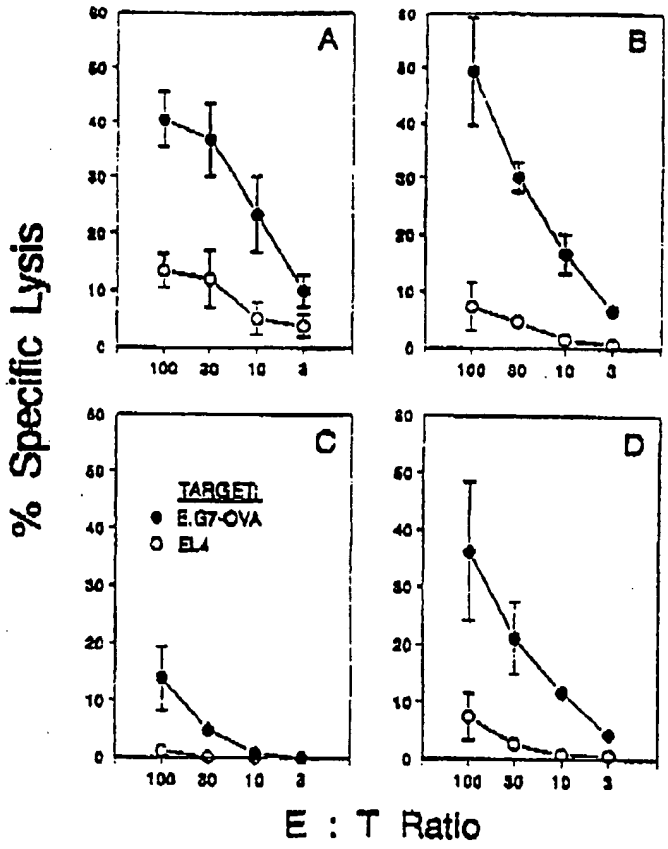


Figure 7

FIGURE 8

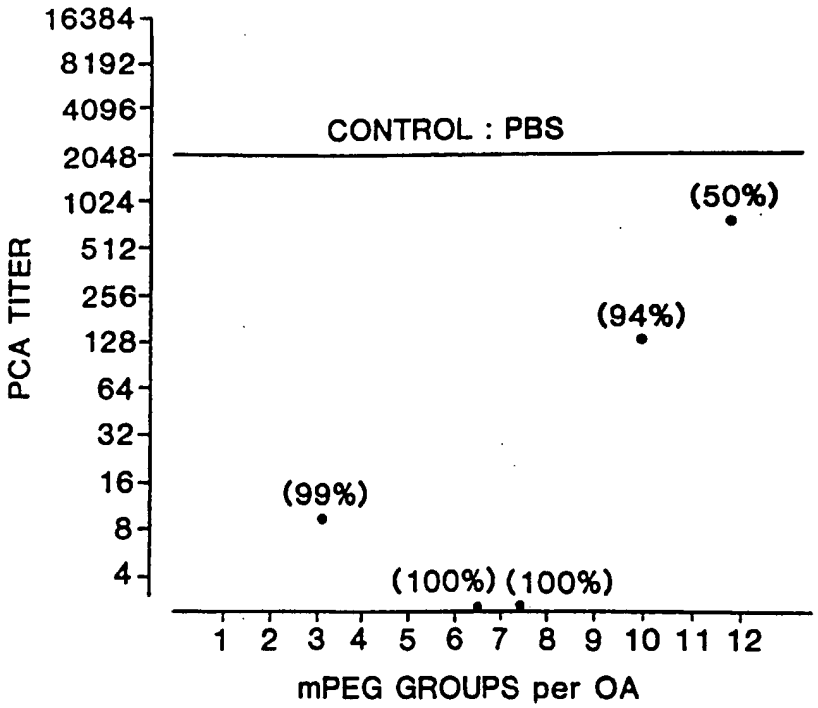


FIGURE 9

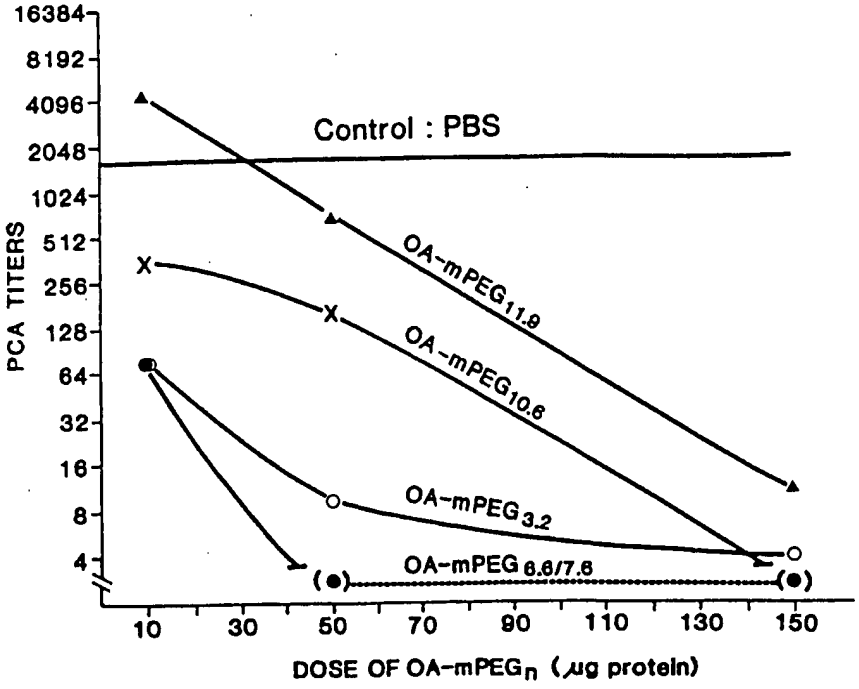


FIGURE 10

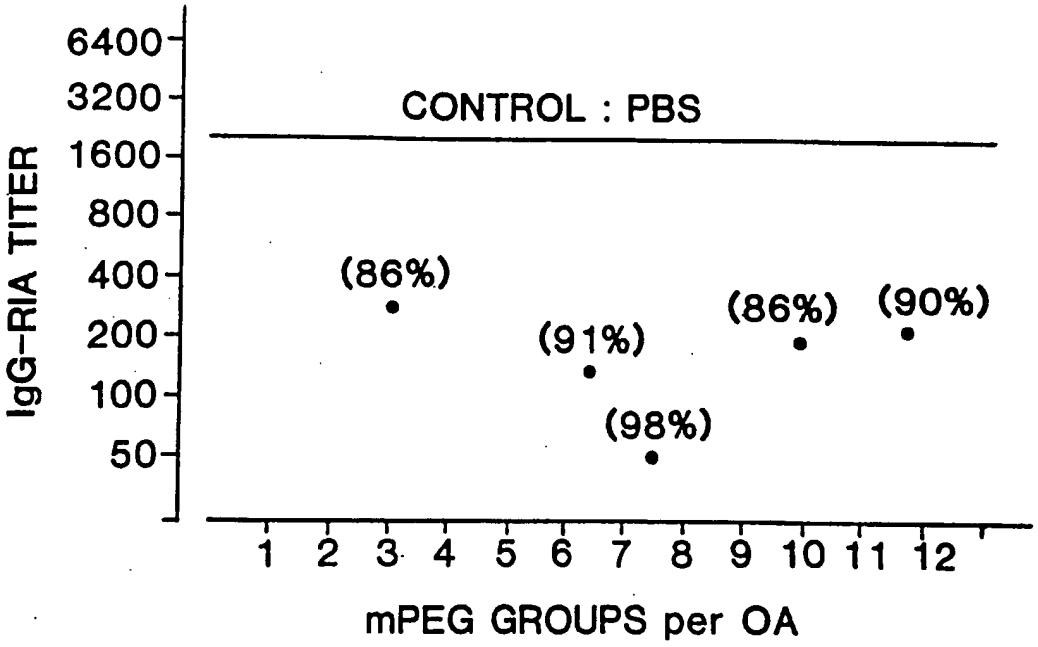
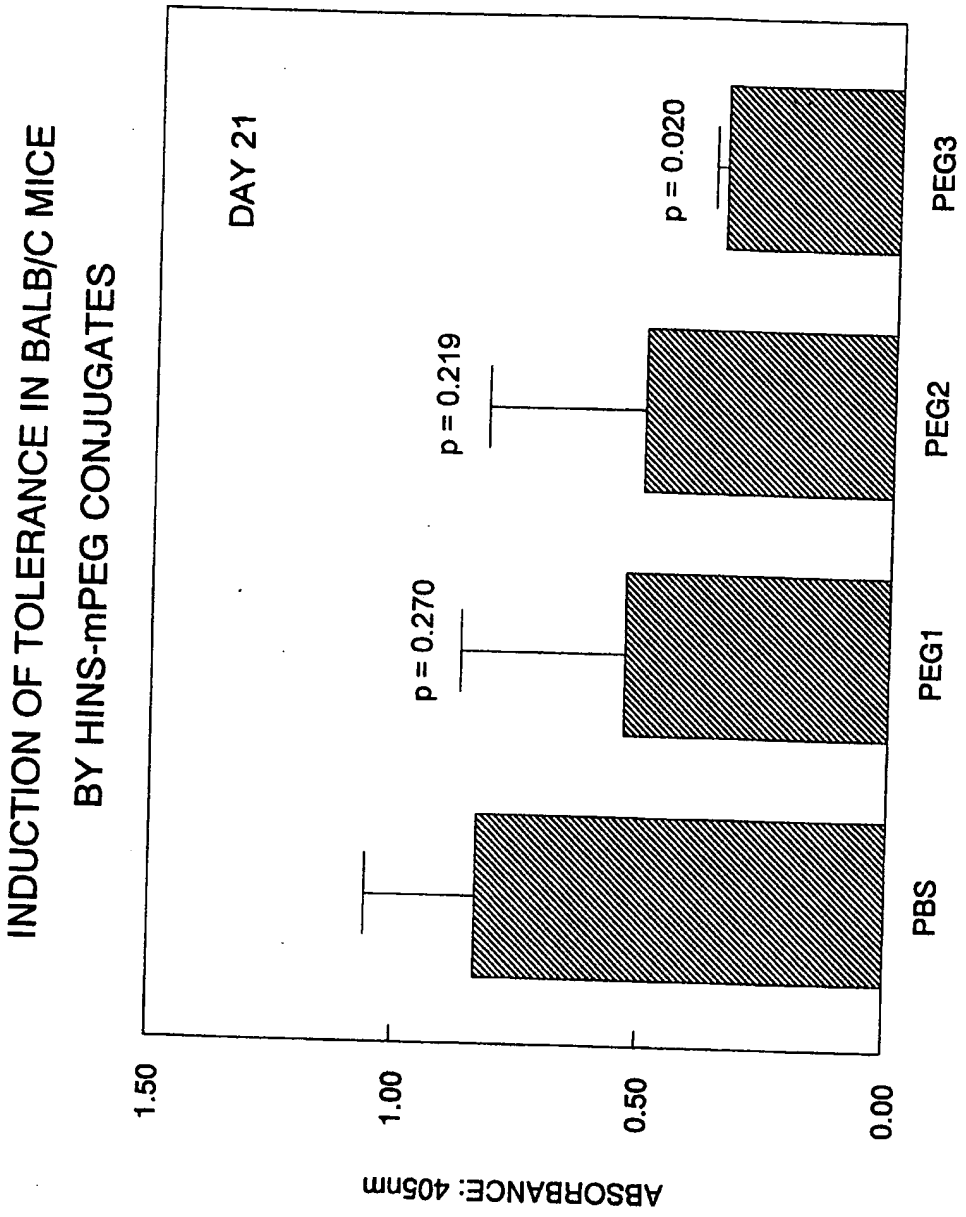

6400
3200
1600
800
400
200
100
50
IgG-RIA TITER
CONTROL : PBS
(86%)
(91%)
(98%)
(86%)
(90%)
1 2 3 4 5 6 7 8 9 10 11 12
mPEG GROUPS per OA

FIGURE 11


INDUCTION OF TOLERANCE IN BALB/C MICE
BY HINS-mPEG CONJUGATES
DAY 21
p = 0.020
p = 0.219
p = 0.270
PEG3
PEG2
PEG1
PBS
1.50
1.00
0.50
0.00
ABSORBANCE: 405nm

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09786

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 39/35, 39/395, C07K 14/00, 16/00
US CL :424/172.1, 178.1, 184.1, 193.1, 805, 810; 530/ 402, 403
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/172.1, 178.1, 184.1, 193.1, 805, 810; 530/ 402, 403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CAPLUS, WPIDS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WILKINSON et al. Tolerogenic Polyethylene Glycol Derivatives of Xenogeneic Monoclonal Immunoglobulins. Immunol. Ltrs. 1987, Vol. 15, pages 17-22, see entire document.	1-27
Y	LANG et al. Suppression of Antibody Responses in Rats to Murine Anti-CD4 Monoclonal Antibodies by Conjugates with Monomethoxypolyethylene Glycol. Immunol. Ltrs. 1992, Vol. 32, pages 247-252, see entire document.	1-27
Y	EP 0496579 A2 (GENERAL HOSPITAL CORPORATION) 29 July 1992, see entire document.	1-27
Y	US 4,261,973 A (LEE et al.) 14 April 1981, see entire document.	1-27.

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
13 JULY 1998

Date of mailing of the international search report
15 SEPTEMBER 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09786

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,126,131 A (DINTZIS et al.) 30 June 1992, see entire document.	1-27

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Manager: Dave Adams

Coaches: Don Bragg, Joe Vollert, Steve Honnert and Steve Dickson

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